DARPin-targeted Chimeric Antigen Receptor T cells: CD4 as a cellular target shows potential to evade HIV latency reservoir

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CD4 as a cellular target shows potential to evade HIV latency reservoir

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1 Introduction

The WHO annual investigation reported that worldwide 36.7 million people, aged 15-45 years, were living with HIV/AIDS in 2016. In some sub-Saharan regions of Africa over 4% of the entire population were infected. One million people died in 2016 due to HIV-related illnesses (WHO 2017).

Combination anti-retroviral therapy (cART) can suppress viral spread and prevent the disease from proceeding (Finzi *et al.* 1999). However, current therapies are targeting viral proteins or inhibiting viral spread by inhibition of cell entry (Pomerantz and Horn 2003). Since the latent reservoir is not specifically eliminated, new viral particles are produced as soon as therapy is terminated (Siliciano *et al.* 2003). Until now, no therapeutic approach to effectively clear the HIV reservoir is available (Pomerantz and Horn 2003).

1.1 HIV and AIDS

AIDS (Acquired Immune Deficiency Syndrome) is a disease caused by the human immunodeficiency virus (HIV). It was first described in the USA in the early 1980s after an accumulation of patients with symptoms of a severely impaired immune system (Friedman-Kien 1981; Siegal *et al.* 1981). In 1983, HIV was identified as the cause for AIDS (Barre-Sinoussi *et al.* 1983).

1.1.1 Acquired Immune Deficiency Syndrome (AIDS)

AIDS is a fatal disease caused by the human immunodeficiency virus (HIV) (Gallo *et al.* 1984; Sarngadharan *et al.* 1984; Schüpbach *et al.* 1984). HIV infects human immune cells, in particular $CD4^+$ T cells. The virus multiplies by using the host cell metabolism and ultimately destroys it. Thereby, the patient's immune system becomes more and more impaired, which makes it susceptible for other viral or bacterial infections. Other common comorbidities are diabetes mellitus, cardiovascular disease, osteoporosis, several types of cancer, renal and liver dysfunction, neurocognitive disease besides a persistent immune dysfunction and inflammation (Chu *et al.* 2017; Holmes *et al.* 2003). In addition to the physical restrictions, patients still have to experience the burden of social contempt and exclusion associated with the stigma of a chronic infection, even now, over 35 years after the disease has been identified (Centers for Disease Control (CDC) 1982). This aversion against HIV^+ patients is mainly caused by the infection routes. HIV is transmitted via different routes. Infections are mostly through mucosal tissues during sexual contact without barrier contraceptives, which affects mostly homosexual men and prostitutes. Another route is the exchange of blood carrying a high viral load, e.g. by using shared needles for drug use but also through untested blood transfusion. The latter can

nowadays be prevented by pre-testing of donor blood. Until effective antiretroviral medication became available, transmission from mother to child during birth was also a very high risk. But, in many developing countries like Sub-Saharan Africa where these treatments are not available for most patients, this threat still remains (Shaw and Hunter 2012).

The general course of the disease is monitored by two factors: The viral load of free infectious particles in the blood serum indicates viral replication, potency and infectivity. The number of $CD4^+$ T cells in the in the blood is a main indicator for the disease progression. It can be divided into three main phases (Figure 1, Deeks *et al.* 2015). The first three weeks after the infection, called eclipse phase, transmitted HIV particles infect the first cells in the new host through their CD4 receptor and CCR5 or CXCR4 co-receptors (Chan and Kim 1998). The viral genome integrates into the host cell and prepares first replication cycles. The following 6 weeks are the acute phase, at which the virus replicates and releases high amounts of newly synthesized particles which, then infect more host cells. The virus thereby establishes a reservoir within the host. In order to establish the best conditions for this invasion, a transitional cellular immune response is induced, which has many benefits for HIV: First, does the activation of T cells increase their expression levels HIV entry receptors, which makes them more susceptible for infection. Second, the release of cytokines induces recruitment and proliferation of more immune cells which serve as hosts for released virus and spread systemically within the patient. During this acute phase, the $CD4^+$ T cell count drops dramatically and the amount of free virus in the serum has its peak. Clinical symptoms are usually similar to an influenza infection. Patients experience fever, enlarged lymph nodes, undefined rash, vomiting or diarrhoea. Occurrence and severity of these symptoms are highly variable and often even not appearing at all. Hence, HIV infections are very often not or misdiagnosed and an important treatment window expires. After the acute phase, the infection passes on to the latent stage. Despite over three decades of intensive research, the mechanisms during this phase are not fully understood. The viral load in the blood serum, meaning free infectious viral particles, decreases significantly. In the beginning, the $CD4^+$ T cells count increases, although it never reaches pre-infection level again. Because the virus has now established a latent viral reservoir in a sufficient number of host cells, it slows down the production of new virions (Ruelas and Greene 2013). This decreases the immune response against the virus itself but also against infected cells, which now simply promote viral replication by cellular proliferation. The latent phase can remain for over ten years, depending on the general health condition of the patient. Because most patients are not aware of their HIV infection, they refrain to use protective measures and unknowingly infect other individuals. During the latent phase, a slight but constant increase of the viral load is accompanied by an analogous drop of $CD4^+$ cells. However, latently infected cells do not differ from uninfected cells, despite the integrated HIV genome. Viral replication can be triggered by activation of infected T cells during an immune response e.g. during an

infection with a different virus, allergic reactions, a cold but also induced by mechanisms within the infected cell itself. A steady-state of various complex virus-host-interactions is established and can continue over many years. How and when these mechanisms are induced and cellular latency-revering factors are involved, remains unknown (Richman 2017). Thus, the establishment of a latent reservoir is the reason why HIV infections can be treated but not cured. At the end of the latent phase, the $CD4^+$ T cells count decreases to a level at which the patient's immune system collapses and they die from minor infections along with organ failure.

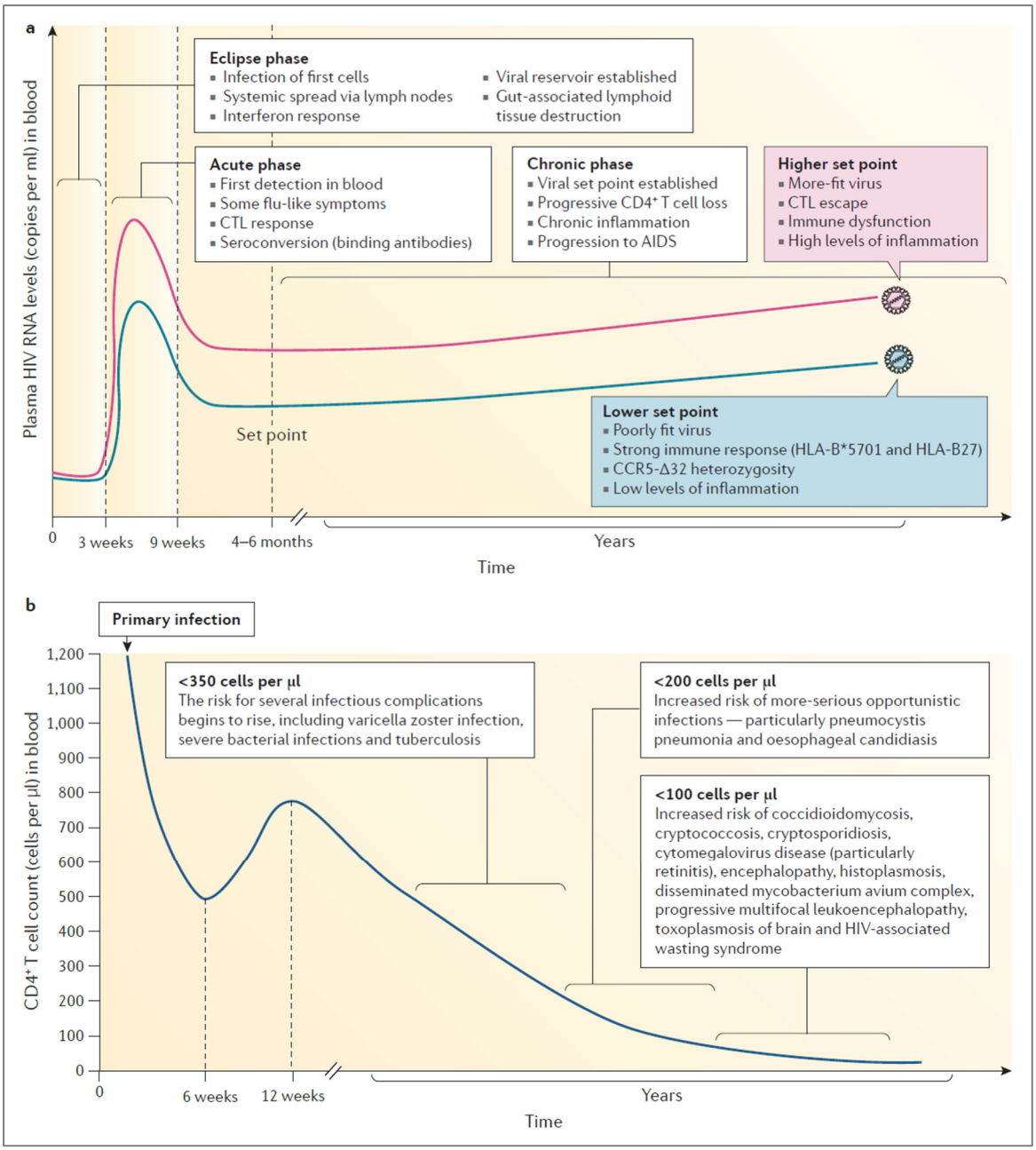

Figure 1: HIV infection and progression of AIDS.

(a) Viral load of infectious HIV particles during disease progression. Within the eclipse phase, transmitted HIV infects target cells in mucosal tissues followed by spread through the lymphoid system. Levels of HIV RNA first become detectable several days post infection. After an exponential increase over a few weeks, HIV peaks and the adaptive immune response achieves partial control. Due to rapid viral escape, HIV antibody responses are largely ineffective. Complex virus–host interactions establish a steady-state level of active and passive viremia. When entering late phase, HIV-mediated destruction of $CD4^+$ T cells leads to immunodeficiency and chronic inflammation. **(b) $CD4^+$ T cell count during AIDS progression.** As the typical $CD4^+$ T cell number declines from 1200-500 to <350 cells per µl, the risk for several infectious complications starts to increase. Within more-advanced disease progression, $CD4^+$ T cell count can drop below 100 cells per µl. HIV-associated immunodeficiency increases the risk of Kaposi sarcoma, certain lymphomas and invasive cervical cancer, amongst others. The US Centers for Disease Control and Prevention defines AIDS on the basis of the presence of HIV infection and either a $CD4^+$ T cell count of <200 cells per µl or an AIDS-defining complication. CCR5 = CC-chemokine receptor 5; CTL = cytotoxic T lymphocyte; HLA = human leukocyte antigen, Figure: Deeks *et al.* 2015.

1.1.2 Human Immunodeficiency Virus (HIV)

HIV is a lentivirus, which belongs to the family of *Retroviridae*. Until now two species, HIV-1 and HIV-2, have been identified of which HIV-1 has a higher pathogenicity in humans. Both strains can be divided into numerous subcategories. It is known that the virus has its origin in the simian analogue SIV (Simian Immunodeficiency Virus). Multiple SIV strains were found in different hosts, such as SIV_{gor} (*gorilla*), SIV_{cpz} (*chimpanzee*) which is the closest to HIV-1 (van Heuverswyn *et al.* 2006) or SIV_{smm} (*sooty mangabey*) the source for HIV-2. In the majority of their natural hosts, SIV is not pathogenic. While in sooty mangabey for example, SIV_{smm} does not cause disease, rhesus macaques develop AIDS. Hence, rhesus macaques (*Macaca mulatta*) is the most common animal model in HIV research (Chen 2018). Several cross-species transmissions from non-human primates to humans have occurred over decades and provided the base for a highly mutagenic pool of virus strains (Wertheim and Worobey 2009). The enveloped virus can be transmitted across mucosal surfaces, by maternal-infant exposure, and blood (Shaw and Hunter 2012). Knowledge about the assembly, functions and interactions of these genes and viral proteins with the host cell is essential to understand the life cycle of the virus and why it has been so difficult to defeat it. The two copies of positive-sense single-stranded RNA contain all nine HIV genes (Figure 2). The translated proteins can be divided into four categories: structural proteins, enzymes, essential regulatory elements and accessory regulatory proteins. Lentiviruses have only one promotor within the LTR. To be able to fit many proteins into a rather short genome, they fall back on multiple ribosomal reading frames and a number of regulatory mechanisms such as post-transcriptional modification. Genes encoding structural proteins are gag, pol, and env, which form the capsid and matrix of the viral particle. Group-specific antigen, gag, forms a precursor polyprotein which is then spliced by the viral protease into the matrix protein p17, the capsid protein p24 and the nucleocapsid protein p7. Env (envelope) encodes also a polyprotein, p160, which is processed within the endoplasmatic reticulum (ER) of the host cell. This results in the glycoprotein gp120, which binds to the CD4 receptor of the host cell, and gp41, a transmembrane protein which connects the envelope to the viral matrix through the surrounding lipid membrane. Thereby, gp41 dimers function as a bridge or tunnel through which the virus can transfer its genome and proteins into the host cell. Viral enzymes are encoded within the pol polyprotein. HIV protease is responsible for the cleavage of gag polyprotein into its subunits. The reverse transcriptase (RT) transcribes the viral RNA into double-stranded DNA which can then integrate into the host genome, transmitted by integrase (IN).

Proteins tat and rev are essential regulatory elements for the viral life cycle. HIV trans-activator tat is involved in the regulation of reverse transcription and regulates the release of virions from infected cells. The second essential regulatory protein is rev, regulator of expression of virion

proteins. It is produced mainly during early infection phase and enriched in the nucleus of the host cell. It consists of two main functional domains. The amino-terminal domain promotes oligomerisation of rev proteins and binds to the reverse response element (RRE) of the viral RNA in the nucleus. The carboxy-terminal domain of rev is activated by cellular proteins through a sequence called NES (nuclear export signal). It forms a complex with the mRNA which is then transported out of the nucleus. After release from rev, these mRNAs are then translated into viral proteins.

Further accessory regulatory proteins are lentivirus protein rapid (vpr), vif, nef and vpu (virus protein out). They are not essentially required for the viral life cycle but function as very potent "life guards" for the virus by inhibiting cellular defence mechanisms. Vpr, serves as a shuttle for nuclear transport. The open reading frame of vpu overlaps with the one of vif and the first exon of tat and the protein is translated from a mono-spliced mRNA. Vpr supports the transport of the pre-integration complex into the nucleus, which is formed of reverse-transcribed dsDNA, integrase, reverse-transcriptase and further structural proteins. By interaction of vpr with nuclear proteins, the pre-integration complex is transported into the nucleus. Furthermore, vpr blocks the cell cycle in G2 phase by inhibition of cyclin B activation and is therefore able to induce apoptosis. HIV-2 and SIV encode an additional protein called vpx which antagonises the cellular restriction factor SAMHD1 (Baldauf *et al.* 2012). Vif plays a key role for the infectivity of successor particles because of its interaction with the cellular proteins APOBEC3. These are cytosine-deaminases which interfere with the synthesis of ssDNA during reverse transcription. By mutating parts of the DNA sequence, APOBEC3 disturbs the homology of the two single strands which are now no longer able to bind to each other and degraded by nucleases. Therefore, APOBEC3 functions as part of the cellular defence against viral infection. Because vif interferes with APOBEC3, it inhibits its activity. Defects in vif result in less potent particles because of more efficient APOBEC3. Since APOBEC3 is not expressed in all cell types at the same level, vif is very useful but not essential for the viral life cycle, although it is very important during infection of peripheral blood lymphocytes. Viral protein U, vpu, is also an inhibitor of the cellular antiviral defence. Vpu exists only in HIV-1 and the chimpanzee version of SIV, SIVcpz. Its amino-terminal domains are embedded into the membrane of the ER and the cytoplasmic membrane, where it prevents tetherin from virion release. In addition, vpu binds to the cytoplasmic domain of CD4 which is then ubiquitinated and degraded. This function may be less important for HIV-2 or SIV, since their affinity to CD4 is much lower and thereby also the possibility for T-helper cells to trigger an immune response against HIV proteins. Negative factor protein, nef, is among the most variable one. Nef is expressed independent of rev by translation of a multi-spliced mRNA. It is therefore expressed during early infection phase and shows a very high variation in between different HIV isolates. One of the many functions of nef is that it is phosphorylated by several other proteins. This phosphorylation can

then induce a signal cascade which leads to increased expression of transcription factors NFκB, AP-1 and NF-AT and chemotactic cytokines, such as MIP-1α and -β. This activates infected T cells, increases the production of viral genome and recruits more T cells which serve as new targets cells for released viral particles. The nef-induced signal cascade also promotes synthesis of the Fas ligand which induces apoptosis of HIV-specific cytotoxic T cells. By interaction with the adaptor-protein complex AP-1 and -2 during the early replication cycle, nef significantly reduces the expression of multiple T cell receptor surface proteins, which are important for the cellular immune response. Reduction of CD4 prevents the viral particle from reinfection of the releasing host cell. Increased expression of env proteins on the cell membrane leads to a stronger release of viral particles. Reduced expression of T cell co-stimulator CD28, inhibits the cellular activation and antigen-specific immune response. Nef induced pathways also lead to downregulation of MHC-I proteins on the surface of antigen-presenting cells. These are just the most prominent examples of how nef regulates expression of cellular proteins. Overall, nef is able to balance their expression according to many surrounding factors of the cellular immune response, always for the benefit of the viral life cycle (Modrow 2010).

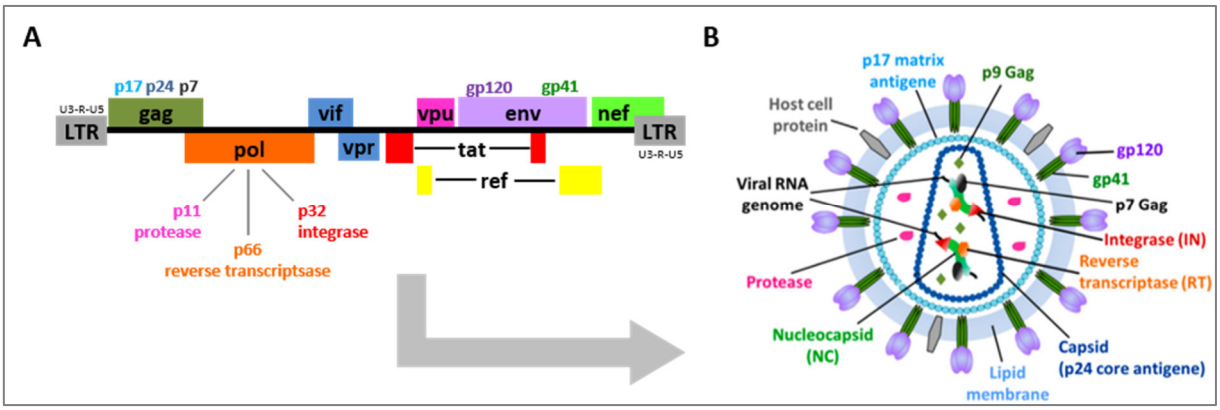

Figure 2: HIV, structure of genome and particles.

(A) The genome of HIV is just under 10kbp long and encodes for nine genes. Each gene is translated into a viral protein. **(B)** HIV proteins assembled to viral particles. Figure modified from Musumeci *et al.* 2015.

The entry process of HIV into the host cell can be divided into five main phases. First, HIV gp120 binds unspecific attachment factors on the host cell (Wilen *et al.* 2012), before env proteins attach to their primary receptor, CD4. During the third phase, gp120 binds to one of the cellular co-receptors CCR5 (R5 tropism) or CXCR4 (X4 tropism). Viruses with the ability to use both co-receptors are named R5X4 HIV. The virus then “screens” the cell surface for a primary entry site which allows productive membrane fusion. This last entry step then enables the virus to fully enter the host cell where RNA and regulatory proteins are uncoated from the viral capsid. Mediated by the reverse transcriptase, viral RNA can now be reverse transcribed into DNA which then enters the nucleus where it is integrated into the host genome. Viral RNA

is then transcribed into mRNA along with the genes of the host cell and transferred back into the cytoplasm. After translation into peptides and proteins, new viral particles can be assembled and released from the host cell (budding and maturation) (Figure 3). Almost every phase within this HIV life cycle can be approached by the latest methods of anti-retroviral therapy. However, a mayor issue hereby is the requirement of intermediate treatment just after or even prior to a potential exposure to the virus. Inhibition of cell attachment, fusion, reverse transcription and integration can potentially prevent a chronic HIV infection. The therapeutic window for this kind of treatment is only a few hours to maximum 2-3 days. However, most patients in this state are not aware that they were exposed to HIV and therefore do not take action in respect (Speil 2009) of a preventive treatment.

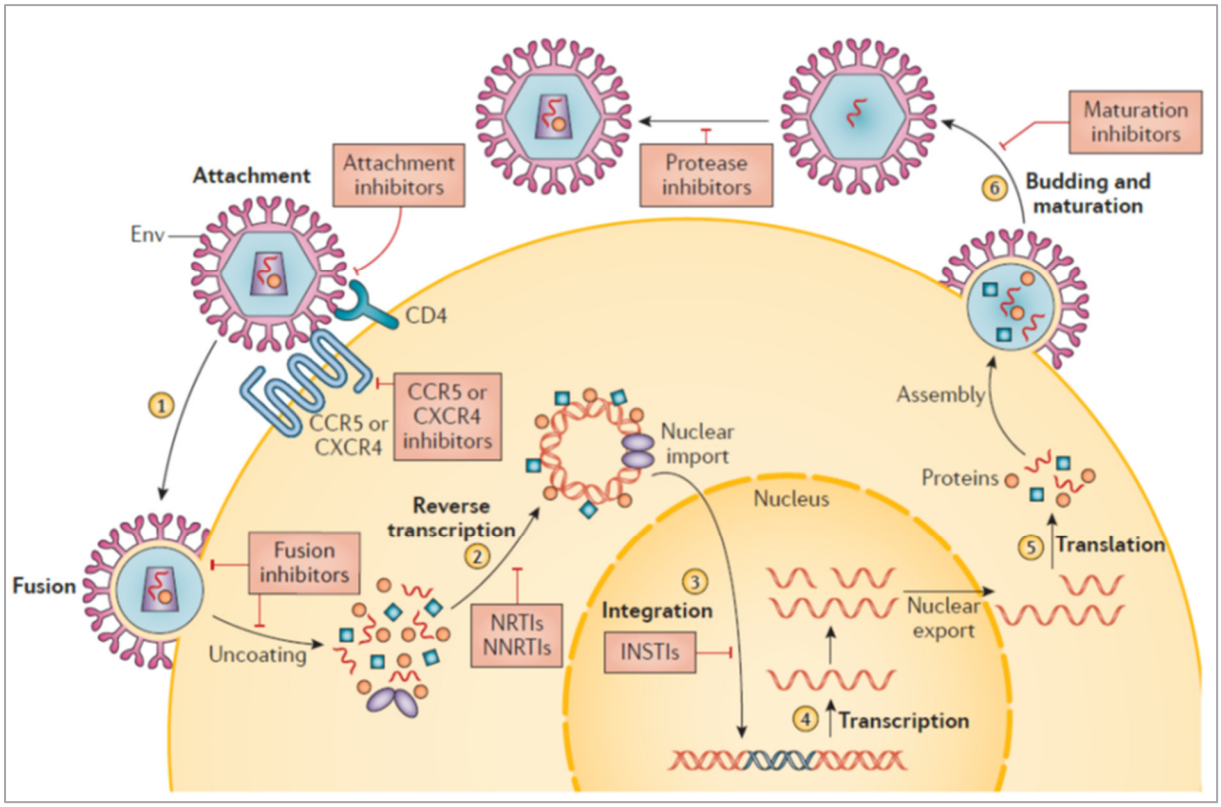

Figure 3: Life cycle of HIV.

HIV enters its target cells via CD4 and either receptors CCR5 or CXCR4 through interaction with envelope (env) glycoprotein (step 1). After fusion and uncoating, the viral RNA is then reverse transcribed into DNA (step 2). The ensuing pre-integration complex is imported into the nucleus, and the viral DNA is then integrated into the host genome (step 3). Mediated by host enzymes, HIV DNA is transcribed to viral mRNAs (step 4). These mRNAs are then exported to the cytoplasm where translation occurs (step 5) to make viral proteins and eventually mature virions (step 6). Each step - HIV entry, reverse transcription, integration and protein maturation - in the HIV life cycle is a potential target for antiretroviral drugs. CCR5 (C-C chemokine receptor type 5), CXCR4 (C-X-C chemokine receptor type 4), INSTI (integrase strand transfer inhibitor), NNRTI (non-nucleoside reverse transcriptase inhibitor), NRTI (nucleoside reverse transcriptase inhibitor). Figure: Deeks *et al.* 2015.

The crucial point of primary infection is reached as soon as the reverse transcribed DNA has been fully integrated into the genome of the host cell. From this time on, maturation and spreading of new viral particles can only be prevented under continuous treatment inhibiting viral maturation and gag-mediated cleavage of the gag polyproteins. As soon as this therapy is stopped, transcribed viral RNA can again be translated into infectious viral particles. Integrated viral DNA forms a so called “reservoir” which can no longer be removed from the host genome (Jordan *et al.* 2003; POZ magazine 2018). HIV infected host cells can then transform into a latent state in which they do not actively produce any virus or transcribe viral RNA. During this HIV latency, the host cell does not cortically differ from any other healthy CD4 memory T cell. During the last decades, HIV research attempted to find a factor or cellular marker which could identify a latently HIV infected cell, but until now without any ground-breaking success (Dahabieh *et al.* 2015; Pillai and Deeks 2017). It was shown recently, that CD32 is widely expressed on most infected cells (Descours *et al.* 2017), but even the total depletion of all CD32 positive cells did not lead to complete elimination of the latent viral reservoir. Stimulation of the CD32 negative cell population led to reduced but not fully inhibited viral replication. One year later, the role of CD32 was revised to be a marker for T cell activation, rather than latent infection (Badia *et al.* 2018). Therefore, latent HIV infection remains a chronic disease of the lymphocytes without a unique marker.

1.1.3 Immune response towards HIV infection

The human immune system is primed to identify and eliminate infected or otherwise abnormal cells (Murphy *et al.* 2012). The first cells recognising a foreign pathogen are unspecific cells of the innate immune system, such as mast cells, phagocytes, macrophages, neutrophils, dendritic cells (DCs), granulocytes or natural killer cells (NK). Uptake of the pathogen is followed by cytokine secretion and recruitment of cells of the innate immune system, to which the foreign peptides are presented. Almost every cell type can serve as an antigen presenting cell (APC), and present peptides of human pathogens such as bacteria or viruses on their major histocompatibility (MHC) complex (Figure 4). Extracellular proteins are presented on MHC-II and intracellular proteins on MHC-I molecules. This cross-presentation enables T cells to recognize and bind to the presented peptides. If a foreign peptide is presented on the MHC-II complex, it is recognised by naïve $CD4^+$ T helper cells (T_{H0}). Depending on the milieu of locally APC-secreted cytokines, the T_{H0} cell differentiates into either a memory helper T cell (T_M), an effector T helper cell ($T_{H1}/T_{H2}/T_{H17}$) or a regulatory/suppressor T cell (T_{reg}). Simultaneous peptide-binding of the T cell receptor (TCR) and the CD8 receptor to the MHC-I complex of an APC initiates activation of the cytotoxic T cell (Figure 4). Thereupon, the cytotoxic T lymphocyte (CTL) or T

killer cell initiates several intracellular pathways, such as the Pi3K pathway, which leads to T cell activation and specific depletion of the antigen-presenting cell:
The intracellular domain of CD45 has an intrinsic phosphatase activity that removes an inhibitory phosphate group on lymphocyte-specific protein tyrosine kinase (Lck), which is thereby activated. Lck can then transfer this phosphorylation to other proteins, such as the cytoplasmic domains of TCR/CD3 or CD28. Binding of a target molecule changes the conformation of the cytoplasmic tail of the CD3 receptor, which allows phosphorylation of immunoreceptor tyrosine-based activation motifs (ITAMs) by Lck. ITAMs are encoded within the CD3zeta chains of TCRs. Thereupon, zeta-chain associated protein kinase (Zap-70) is recruited to the phosphorylated TCR/CD3 complex where it becomes activated, promoting a cascade of phosphorylation events within diverse signalling pathways. The most important targets for ZAP-70 are the transmembrane protein linker for the activation of T cells (LAT) and the cytosolic lymphocyte cytosolic protein 2 (SLP-76). Formation of a complex by adaptor proteins LAT and SLP-76 leads to reorganization of effector molecules in a way that allows activation of multiple signalling pathways. These signalling pathways result in promotion and activation of transcription factors such as NF-κB and NFAT(Smith-Garvin *et al.* 2009), which leads to a high increase of transcription of cytokines, such as IFN-γ, IL-2, IL-6 and TNFα. Additional binding of costimulatory receptors such as CD28 to CD80 or CD86 or CTLA4 to B7-1 or -2, mainly through Pi3K pathway, can then initiate specific T cell killing (Murphy *et al.* 2012). During this process, the T cell forms an immunological synapse around the site of antigen recognition which is fully isolated from the surrounding milieu. It then releases Perforin and cytotoxic granules such as Granzyme B into this synapse. Perforin forms pores or channels into the membrane of the target cell which enable cytotoxic granules to enter the cytosol. There, Granzyme B can cleave and activate initiator caspases 8 and 10, and executioner caspases 3 and 7 which trigger apoptosis (Afonina *et al.* 2010).

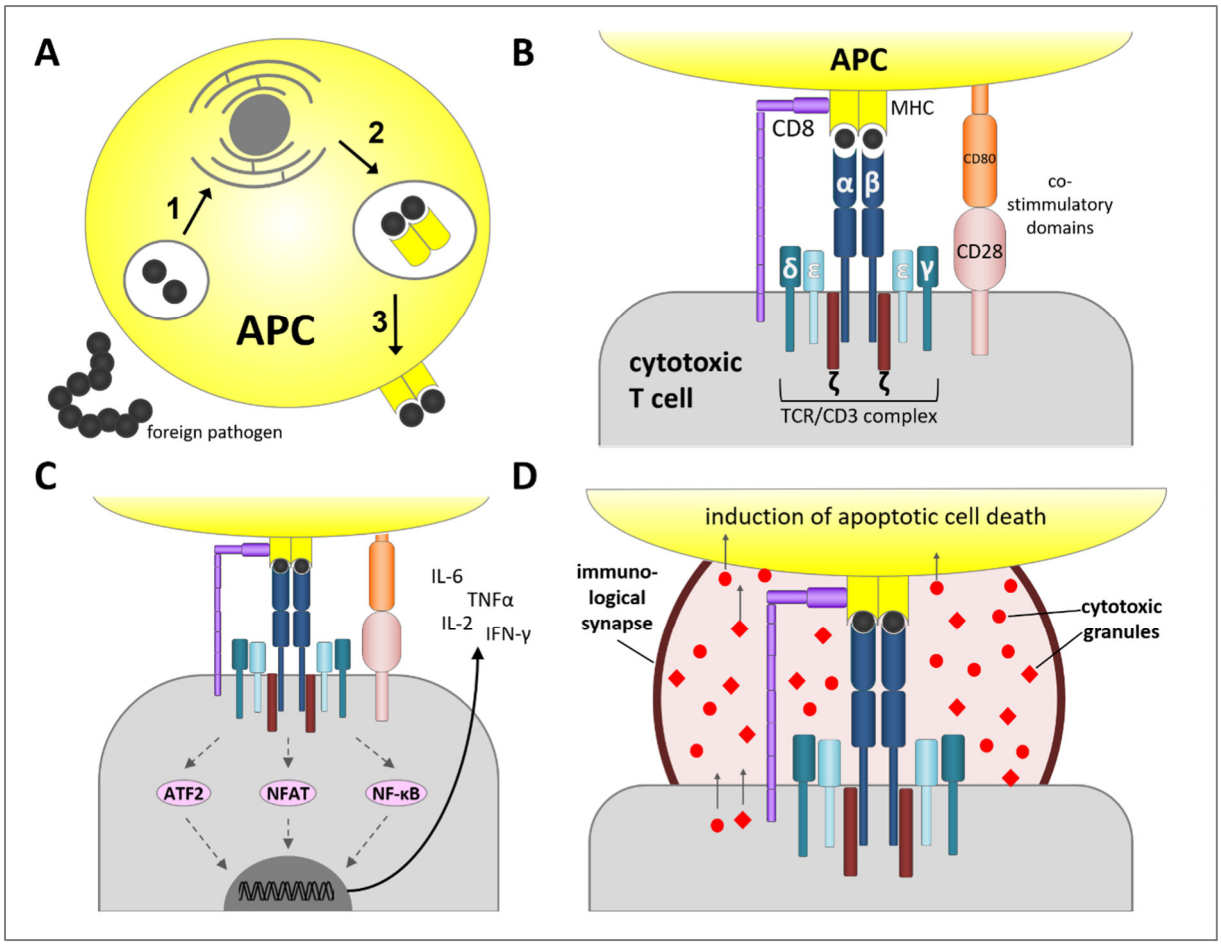

Figure 4: Antigen-presentation to cytotoxic T cells.

(A) Uptake and processing of foreign pathogens by antigen presenting cells (APCs). After endocytic uptake, foreign peptides are degraded into smaller units by proteases in the cytosol (1). (2) Via heat shock proteins, processed peptides are distributed to the endoplasmatic reticulum (ER) in which they are loaded onto the MHC-I complex. From the ER, the MHC-I-peptide complex is then transferred to the cell surface in exocytic vesicles (3). **(B)** Processed peptides of a foreign pathogen are presented on the MHC-I complex to the T cell receptor (TCR) complex of a cytotoxic T cell. Binding of the CD8 receptor to MHC-I and co-stimulatory domains, such as CD28, trigger the T cell activation. **(C)** Several signalling pathways within the T cells are activated and transcription factors initiate transcription of numerous cytokines, which are then secreted by the T cell. **(D)** An immunological synapse is formed around the site of antigen recognition. Cytotoxic granules, such as Granzyme B and Perforin are released into the synapse leading to apoptotic cell death of the APC.

During an infection with HIV, these immune mechanisms are taking place as well. However, they are not efficient in clearance of the viral particles, infected cells or prevention of a latent viral reservoir (Perreau *et al.* 2013). On the contrary, HIV takes advantage of this immune reaction using activated $CD4^+$ T cells, especially resting memory T cells (T_{RM}), as a primary target (Crooks *et al.* 2015; Schnittman *et al.* 1990). Several factors within the HIV life cycle help the virus to survive the immune response. One example is that the viral reservoir is established before initiation of an immune response. During the first phase of infection, the so-

called “eclipse phase”, no viral RNA or proteins can be detected in the plasma for 1-3 or sometimes even up to 10 days (McMichael *et al.* 2010). This makes part of the virus invisible towards an immune-defence. By the time at which HIV is recognized, cytokines are released and immune cells are activated, the HIV genome has already integrated into host cells and produces more viral particles. Furthermore, are immune cells themselves the primary target for HIV. Activation of $CD4^+$ T cells is even an advantage for HIV, since more provirus is transcribed and more potential target cells are recruited and activated – optimal conditions for viral spread. As shown in Figure 1, the number of $CD4^+$ T cells in the blood decreases strongly during the peak of viral replication in the acute phase of infection. Direct cytopathic effect depletes the major amount of T cells after they released high amounts of virus. It was found that during the acute phase, up to 20% of $CD4^+$ T cells in the gut are infected and 80% depleted (Perreau *et al.* 2013). This leads to a situation in which cytotoxic T lymphocytes (CTLs) do not find enough antigen-presenting target cells to be sufficiently activated. Therefore, CTLs do not release enough cytokines to proliferate and achieve an effective depletion of infected cells. Another reason for the lack of an efficient immune response towards HIV is the high mutation rate of the viral proteins (Cuevas *et al.* 2015). However, it has been shown that activated HIIV-specific CTLs are a requirement for a significant depletion of HIV-infected cells (Shan *et al.* 2012). During the initial infection, already multiple different variants of the virus are transmitted to which the adaptive immune system needs to respond. HIV-specific T cells are thereby only able to cover a small proportion of the virus. The same effect has been observed for secreted neutralising antibodies (nAbs). They can be detected around 3 months post infection (Frost *et al.* 2008). Most nAbs are binding to HIV env, which shows a very high mutation rate (Haddox *et al.* 2016). Only a small proportion of patients (around 20%) is able to generate neutralising antibodies covering a broad variety of different mutants. However, even these broadly neutralising antibodies failed to control viremia in the long term (Conley *et al.* 1994; Nelson *et al.* 2007; Moore *et al.* 1995). Another cause for the failure of the humoral immune response has also been observed in form of abnormal activation, differentiation and exhaustion of B cells during HIV infection (Lane *et al.* 1983; Moir and Fauci 2008; Moir *et al.* 2008).

Taken together, HIV is able to evade the innate and adaptive human immune system through the time course of replication, infection and cell depletion as well as through high mutation rates and manipulation of the cellular response of the host.

1.1.4 Current methods in HIV therapy

Today, the most efficient treatment for patients infected with HIV is combination antiretroviral therapy (cART). cART is a patient-specific combination of multiple drugs interfering with different states of the HIV life cycle (Figure 3). Progression of the disease can thereby be suppressed as long as the patient remains under close monitoring and adjusted treatment. In 2018, around 35 different FDA-approved drugs are on the market for treatment of HIV infection (Table 1) (FDA 2018). All drugs categorised according to the phase of the viral life cycle they are acting on.

Among the first group of drugs approved for treatment of HIV infection were the nucleoside reverse transcriptase inhibitors (NRTIs). These analogues of native nucleosides or nucleotides have the preference to incorporate into HIV DNA during reverse transcription from viral RNA. Synthesis of HIV encoding DNA is thereby disrupted. Today, most first-line treatments still contain two different nucleotides or nucleosides and combined with a third component targeting another enzyme within the HIV cycle. However, the majority of NTRIs needs to be applied at least once a day which increases the risk of neglecting single doses. Furthermore, many NRTIs showed side effects of variable severity. For example, did tenofovir impair renal and bone function in some patients (Tourret *et al.* 2013). Abacavir caused severe allergic reactions in patients expressing HLA-B*5701 (Baniasadi *et al.* 2016), whereby they require sensitive prescreening. No longer recommended as a first-line treatment by the WHO are zidovudine and stavudine, after anaemia, neuropathy, hepatic steatosis, lactic acidosis and lipoatrophy have been observed during treatment (Deeks *et al.* 2015). Improved versions of NRTIs bearing less side effects have mostly been less effective, even though some promising candidates are investigated further (Cahn *et al.* 2014).

Besides nucleoside RT inhibitors, another class of nonnucleoside reverse transcriptase inhibitors (NNRTIs) is available for HIV treatment. Instead of targeting the viral DNA as a product of reverse transcription, NNRTIs bind to a pocket near the active domain of the reverse transcriptase itself. This causes a conformational change of the enzyme, making it incapable to perform reverse transcription. NNRTIs appear to be very effective and cheap in production. Apart from minor toxicity in the CNS and a potentially increased risk of depression observed in some patients, NNRTIs are also comparatively safe. Nevertheless, have first regimen drugs such as nevirapine ranked down, after severe hepatotoxicity and rash had been reported in patients with higher CD4 T cell counts. Whereas rilpivirine was well tolerated but also less efficient in patients with high viral loads (Cohen *et al.* 2014).

Protease inhibitors prevent the cleavage of HIV polyproteins and with that, the maturation of viral proteins, required for the assembly of mature HIV particles. Protease inhibitors are mostly used in combination with NRTIs as so-called pharmacological boosters. An increased risk of

cardiovascular disease and mild gastrointestinal symptoms have been reported during treatment with some protease inhibitors, whereas others caused less side effects but also less efficacy. To date, darunavir is the only protease inhibitor recommended as a first-line treatment by the US Health and Human Services (AIDSinfo 2018; Deeks *et al.* 2015; AIDSinfo 2018).
So far, the only HIV entry inhibitor is maraviroc. Maraviroc affects exclusively virus with CCR5 tropism. It is not applicable for patients bearing HIV using CXCR4 as a co-receptor. Enfuvirtide binds directly to HIV particles preventing their fusion to the host cell. However, production of enfuvirtide is very cost intense and must be applied by an injection twice a day.
Integrase stand transfer inhibitors are within the most potent and effective class of anti-HIV drugs. Besides some minor impact on kidney function, they appear to be safe and well-tolerated. They block integration of reverse-transcribed viral DNA into the genome of the host cell. Dolutegravir, raltegravir and elvitegravir have been shown similar efficacy along with minimal side effects (Raffi *et al.* 2013; Lennox *et al.* 2014; Rockstroh *et al.* 2011). In combination with cobicistat, a booster which prevents rapid clearance by the kidney, integrase stand transfer inhibitors are included in most recommended first-line treatments (U.S. Department of Health and Human Services 2018).
Most ART strategies use a combination of three or more drugs to assure efficacy of the therapy. Because over years of treatment, the majority of patients develops resistances to certain components or experiences side effects, drugs have to be altered accordingly. Pivotal if a person will be irreversibly infected with HIV is the time point of treatment. If integration of reverse transcribed viral DNA into the host genome can be fully prevented, a viral reservoir cannot be established. However, this treatment window is effective for a maximum of 72h and is recommended to continue for at least four weeks (DeGruttola *et al.* 2000). This form of post-exposure prophylaxis (PEP) is often held for groups of enhanced risk of infection, such as medical or laboratory staff (occupational health), as well as sex related or drug users (non-occupational health). When applied immediately after HIV exposure, PEP can prevent fusion of viral particles with the host cell, reverse transcription of viral RNA or integration of the provirus. People at an even higher risk of HIV infection can also use these drugs as pre-exposure prophylaxis (PreEP). Although studies involving homo- and heterosexual couples of which one partner is positive for HIV and the other one is HIV negative, showed controversial results (Grant *et al.* 2010; Baeten *et al.* 2012), preexposure prophylaxis is recommended for these high risk groups. However, looking at the high costs and the occasionally severe side effects, this form of HIV prevention is cannot be considered for the overall population. For a general worldwide protection from HIV infection, the development for a safe, effective and low-cost vaccination strategy is required. Because of the failure of the immune response and the high mutation rate of HIV, this goal has appeared to be unattainable (McMichael and Hanke 2003; Zhang and Lewin

2018). To date, cART has turned HIV infection from an acute lethal into a chronic disease but without the perspective of an ultimate cure.

Table 1: FDA approved drugs used in the treatment of HIV infection.

(Status as of 12.04.2018, FDA 2018)

Brand	Generic Name	FDA approval
Multi-class Combination Products		
Atripla	efavirenz, emtricitabine and tenofovir disoproxil fumarate	July 2006
Complera	emtricitabine, rilpivirine and tenofovir disoproxil fumarate	Aug 2011
Evotaz	atazanavir sulfate, cobicistat	Jan 2015
Prezcobix	cobicistat, darunavir ethanolate	Jan 2015
Stribild	elvitegravir, cobicistat, emtricitabine, tenofovir disoproxil fumarate	Aug 2012
Nucleoside Reverse Transcriptase Inhibitors (NRTIs)		
Combivir	lamivudine and zidovudine	Sep 1997
Emtriva	emtricitabine, FTC	July 2003
Epivir	lamivudine, 3TC	Nov 1995
Epzicom	abacavir and lamivudine	Aug 2004
Hivid	zalcitabine, dideoxycytidine, ddC (no longer marketed)	June 1992
Retrovir	zidovudine, azidothymidine, AZT, ZDV	March 1987
Trizivir	abacavir, zidovudine, and lamivudine	Nov 2000
Truvada	tenofovir disoproxil fumarate and emtricitabine	Aug 2004
Videx EC	enteric coated didanosine, ddI EC	Oct 2000
Videx	didanosine, dideoxyinosine, ddI	Oct 1991
Viread	tenofovir disoproxil fumarate, TDF	Oct 2001
Zerit	stavudine, d4T	June 1994
Ziagen	abacavir sulfate, ABC	Dec 1998
Nonnucleoside Reverse Transcriptase Inhibitors (NNRTIs)		
Edurant	rilpivirine	May 2011
Intelence	etravirine	Jan 2008
Rescriptor	delavirdine, DLV	April 1997
Sustiva	efavirenz, EFV	Sep 1998
Viramune (Immediate Release)	nevirapine, NVP	June 1996
Viramune XR (Extended Release)	nevirapine, NVP	March 2011
Protease Inhibitors (PIs)		
Agenerase	amprenavir, APV (no longer marketed)	April 1999
Aptivus	tipranavir, TPV	June 2005
Crixivan	indinavir, IDV,	March 1996
Fortovase	saquinavir (no longer marketed)	Nov 1997
Invirase	saquinavir mesylate, SQV	Dec 1995
Kaletra	lopinavir and ritonavir, LPV/RTV	Sep 2000
Lexiva	fosamprenavir Calcium, FOS-APV	Oct 2003

Brand	Generic Name	FDA approval
Norvir	ritonavir, RTV	March 1996
Prezista	darunavir	June 2006
Reyataz	atazanavir sulfate, ATV	June 2003
Viracept	nelfinavir mesylate, NFV	March 1997
Fusion Inhibitors		
Fuzeon	enfuvirtide, T-20	March 2003
Entry Inhibitors - CCR5 co-receptor antagonist		
Selzentry	maraviroc	Aug 2007
HIV integrase strand transfer inhibitors (INSTI)		
Isentress	raltegravir	Oct 2007
Tivicay	dolutegravir	Aug 2013
Vitekta	elvitegravir	Sep 2014
Isentress	raltegravir	Oct 2007
Tivicay	dolutegravir	Aug 2013
Vitekta	elvitegravir	Sep 2014

1.1.5 Latent HIV reservoir

As discussed in Section 1.1.4, combination ART has saved the lives of millions of HIV-infected patients by inhibiting and suppressing the viral life cycle. However, these drugs have shown severe side effects, impairing the expected life span (Hoffmann and Kamps 2007). Gastrointestinal and renal problems, liver toxicity, neurological and haematological side effects, allergic reactions, osteopenia/osteoporosis as well as cardiovascular diseases and diabetes are just some examples of comorbidities during cART. Consistent escape mutations promote the development of drug resistance (Harrigan *et al.* 2005; Mosier 2007; Phillips *et al.* 2005). The suppression of viral replication can only be maintained, as long as retroviral therapy continues (Ho *et al.* 2013). As soon as the treatment is stopped, viral replication and disease progression are reverted (Henrich *et al.* 2017; Finzi *et al.* 1999; Siliciano *et al.* 2003). In some cases, viral replication persists despite extensive cART (Martinez-Picado and Deeks 2016). The reason for this persistence of HIV in the host is the latent reservoir (Jordan *et al.* 2003). Around 1-3 days after infection, the reverse-transcribed viral DNA integrates into the host genome and is thereby irreversibly embedded (Chun *et al.* 1998). From this moment, the virus is capable of initiating new replication cycles, as long as the cell persists (Wong *et al.* 1997). An HIV infected cell can be in an active state at which high amounts of viral particles are synthesised and released, mainly during acute infection. After 9-12 weeks, transcription of the provirus is significantly decreased and can remain quiescent for many years. Intermediate states between these two conditions are continuously variable. The stimuli and mechanisms which promote or reverse HIV latency are still not fully understood (Ruelas and Greene 2013; Dahabieh *et al.* 2015). The variety of different host cell types are already indicating, that not just one exclusive

trigger or pathway is responsible for reversion of HIV latency, although a number of heterogeneous cellular and viral mechanisms have been identified to regulate HIV latency (Dahabieh *et al.* 2015).

Besides the variation in primary infecting virus mutants, the types of targeted host cells vary as well. Giving CD4 entry receptor as the common denominator, HIV infects macrophages, myeloid, plasmacytoid and follicular dendritic cells (mDCs, pDCs, fDCs), Langerhans cells and above all, $CD4^+$ T cells. All these cells contain more or less different intracellular proteins and enzymes and induce intracellular pathways to a different extent. In conclusion, several factors must be involved in the host pathogen-interactions during HIV latency. Nevertheless, two major latent reservoirs have been identified (Chomont *et al.* 2009). Resting central memory T cells (T_{CM}) and translational memory T cells (T_{TM}) showed different decay rates during ART. Patients who started therapy early after infection had high $CD4^+$ T cell counts harbouring only a small viral reservoir mainly in T_{CM}. These T_{CM} proliferate at extremely low levels but persist for decades. In patients who received ART at a much later state of infection and had low $CD4^+$ T cell counts, the latent viral reservoir was found mainly in T_{TM}. A slight but constant immune activation in these patients, causes secretion of IL-7, supporting proliferation of these T_{TM} cells (Hodge *et al.* 2011; Vandergeeten *et al.* 2013). In general, a drop of plasma RNA levels of 99% is achieved during the first two weeks of ART. After this initial phase, a much slower progress is observed, involving death of macrophages, although they appear much more resistant to cytopathic effects than other cell types. Although infected mDCs do not seem to play a major role in the latent reservoir, they were shown to be able to survive over more than 45 years (Popov *et al.* 2005). Monitoring viral persistence has brought up many contrary but also some corresponding observations, involving all phases of the viral life cycle. Only a few of them can be described here. Orientation and locus of viral integration are already pointing the way of how actively the provirus will be transcribed later on. Orientation of the provirus parallel to the host gene promotes a higher transcription rate while anti-parallel orientation occurred in latent cells (Han *et al.* 2008). Also, de-condensed integration sites which provide a good accessibility for transcription factors are more likely to cause a high basal HIV promoter activity than a transcriptionally repressive environment (Hughes and Coffin 2016). If for example a HIV promoter is occlusive, the RNA polymerase II (Pol II) starting from an upstream host promoter will simply read through the HIV genome, causing displacement of necessary transcription factors from the HIV genome (Greger 1998). Whereas convergent transcription of host and viral genome often leads to collision of the two RNA Pol II complexes and termination of transcription from both or the weaker promoters (Lewinski *et al.* 2005).

Manipulation of transcription factors has likewise an essential impact on HIV transcription. During T cell activation, TFs such as NFκB, NFAT, Sp1 and AP1 are translocated from the cytoplasm into the nucleus where they find multiple binding sites within the 5'-LTR of HIV. In resting

$CD4^+$ T cells, NFκB and NFAT are detained in the cytoplasm, unable to migrate into the nucleus. HIV transcription is thereby influenced by the activation status of the host cell (Kinoshita *et al.* 1998). Attempts to reactivate resting latently infected cells with cytokines such as IL-2, IL-6, IFN-γ and TNFα as well as anti-CD3 antibodies have been undertaken. Lafeuillade *et al.* 2001 could achieve viral rebound after termination of ART along with IL-7 and IFN-γ. However, this reactivation also induced secretion of IL-7 and this in turn promotes proliferation of T_{CM}, the major HIV reservoir. It was therefore concluded, that an immune reactivation is likely to cause more damage than benefit to the patient. In addition to initiation of transcription, elongation is influenced as well. Elongation of HIV RNA is highly dependent on the HIV tat protein. By binding to the trans-activating response element (TAR), an RNA stem-loop structure, tat recruits positive elongation factor-b (pTEF-b), a promotor of HIV transcription. pTEF-b together with cyclin-dependent kinase 9 (CDK9) (Wei *et al.* 1998) phosphorylates the C-terminal domain of RNA Pol II and thereby enhances its productivity (Kim *et al.* 2002). In addition, pTEF-b phosphorylates DSIF and NELF (DRB sensitivity inducing factor and negative elongation factor) (Yamaguchi *et al.* 2013). NELF is thereby removed from RNA Pol II and phosphorylated DSIF acts as a positive elongation factor (Fujinaga *et al.* 2003; Ivanov *et al.* 2000). Via a feed-forward loop, more tat is translated and accumulates in the host cell where it further promotes HIV transcription as described (Dahabieh *et al.* 2015). Chromatin modification is another process known to influence HIV expression. Gene expression is highly dependent on the chromatin structure, which in turn is altered by epigenetic factors. In heterochromatin, the compact DNA structure is bound to nucleosomes through which HIV promoters can be blocked and HIV transcription is suppressed. In contrast, the relaxed and transiently open euchromatin encourages transcription. Acetylation leads to relaxed chromatin, accessible for TFs, whereas deacetylation induces compression of the chromosomal DNA. Changes of chromatin condensation can be mediated through Acetyl Coenzyme-A (Acetyl-CoA). Histone acetyltransferases (HATs) and deacetylases (HDACs) catalyse acetylation and deacetylation respectively. van Lint *et al.* 1996 showed that transcription of HIV-1 could be increased by inhibition of HDAC. Enhancement of transcription could thereby increase the accessibility for other therapeutics. As mentioned in Section 1.1.4, HDACs do not impact transcription of HIV exclusively, but many other processes (Glaser *et al.* 2003). Besides deacetylated histones, another characteristic of heterochromatin is the high methylation of the DNA. DNA of HIV 5'-LTRs was found to be hypermethylated in latently infected cells and thereby prevent binding of TFs such as NFκB and Sp1 (Bednarik *et al.* 1991; Kauder *et al.* 2009). However, this GpG methylation within 5'-LTR was identified to prevent reactivation and to be responsible for maintenance of HIV latency, but not for its establishment (Blazkova *et al.* 2009). Furthermore, Lusic *et al.* 2013 have shown, that PML bodies (promyelocytic leukaemia) interact with the latent provirus by recruiting histone methyl transferase 9a, which potentially represses HIV-LTR.

All these observations enable just a small extract of the many factors inducing and reverting HIV latency. Proteins, enzymes and the pathways they are involved in do influence each other, resulting in a very complex matrix of mechanisms of which the majority is still unknown (Richman 2017). A therapy able to overcome HIV latency remains unrivalled.

1.2 Adoptive cell therapy

As explained in Section 1.1.3, the human immune system is unable to sufficiently defend infection with HIV as it does for other viruses. In order to recognise a pathogen as foreign, it needs to be presented on the MHC-complex and trigger a T cell response which is potent enough to eliminate all infected cells. In addition, humoral and cellular immune response need to induce other factors, such as recruitment and activation of helper cells by cytokine secretion. One of the strategies HIV uses to evade the human immune response is to induce downregulation of MHC in the host cell. In order to increase the potency of the cellular immune response towards HIV infected cells, techniques were developed arming immune cells to specifically deplete their target cells without the need of MHC presentation.

1.2.1 Chimeric Antigen Receptors (CARs)

Scientists developed a transgenic receptor which combines the specific and precise features of cytotoxic T cells with MHC-independent binding properties of monoclonal antibodies. In order to circumvent this MHC restriction but still be able to exploit the specific killing properties of cytotoxic T cells, so called "**C**himeric **A**ntigen **R**eceptors (CARs)" were designed (Eshhar *et al.* 1993). These artificial receptors are hybrids consisting of the intracellular chains of a TCR fused to the extracellular binding domain of a monoclonal antibody (Figure 5). When expressed on the surface of a T cell, CARs recognize their respective antigen, regardless if self or foreign, independent of MHC presentation. Thereby, the conformational change of the binding domain transmits a signal to the intracellular CAR domains where the same signalling cascades are initiated as during regular T cell activation due to MHC antigen presentation. Activation of the intracellular pathways leads to T cell activation and initiation of the mediated killing mechanism described in Section 1.1.3. Thus, CAR-T cells combine the specificity of a monoclonal antibody with the cytotoxicity of a T cell without the need of MHC presentation. Moreover, the cytotoxicity is limited to antigen expressing target cells, whereas conventional chemotherapies target all cell types whereby they also affect healthy tissues.

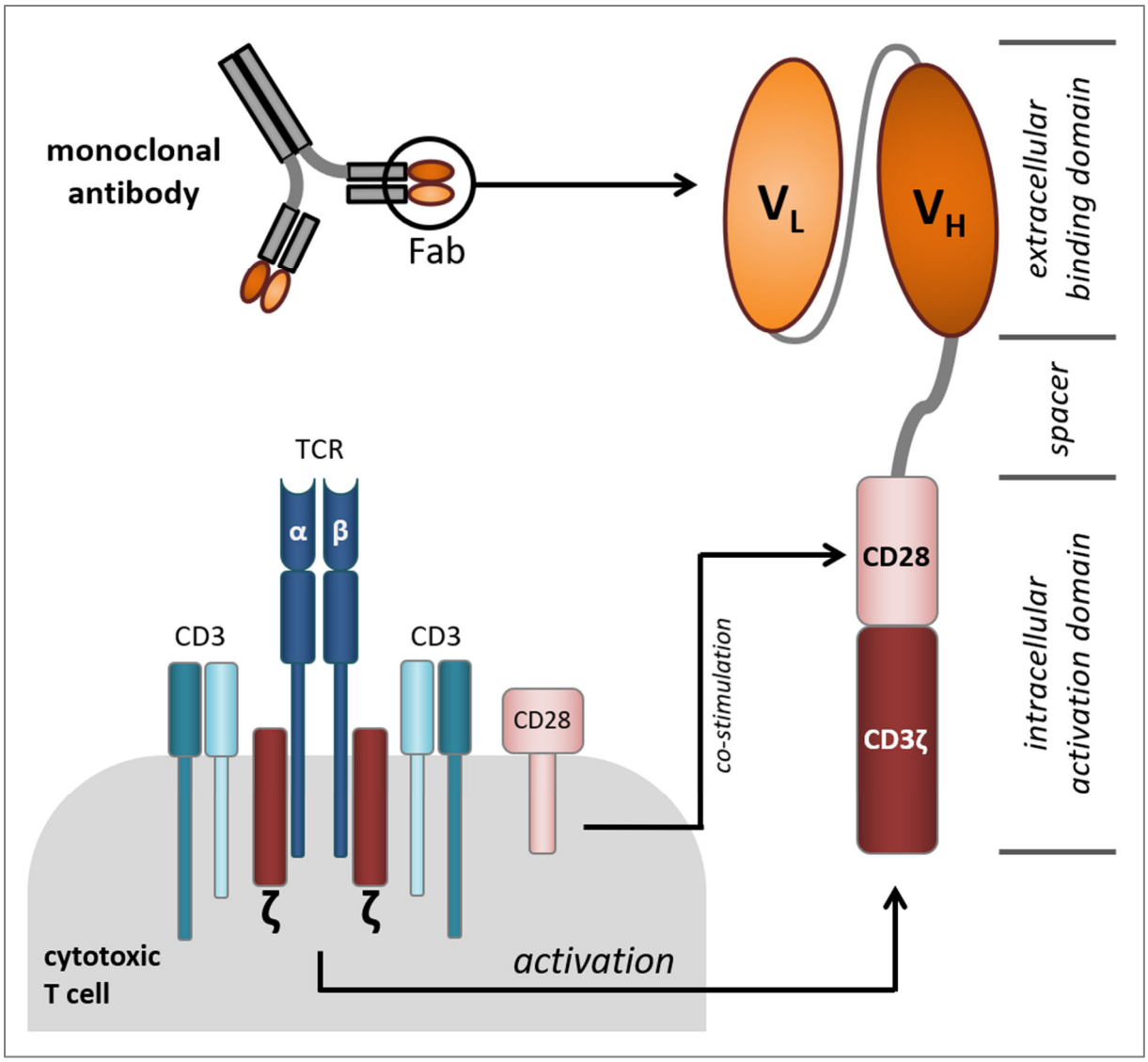

Figure 5: Structure of a Chimeric Antigen Receptor (CAR).

Extracellular CAR binding domains are mainly derived from the Fab fragment of a monoclonal antibody which is fused to the intracellular activation domain by a spacer. For second generation CARs, the activation domain mostly consists of a primary part such as the CD3zeta chain fused to CD28 for transmission of the co-stimulatory signal.

The first generation of CAR-T cells was designed of a CD4 domain, capable of binding HIV env protein, linked to an intracellular CD3zeta chain to induce activation of the redirected T cell (Eshhar *et al.* 1993). Although these adopted T cells were shown to be safe, stable and active in the patients over decades, they were not capable of disease control (Scholler *et al.* 2012). While the CAR-T cell approach went out of the focus of HIV research, the technology sparked interest in oncology. Cancer raises a similar caveat, namely insufficient recognition by the immune system due to lack of antigen presentation. Tumour cells do not express peptides which are recognized as “foreign antigens”. Therefore, their presentation does not trigger immune cell activation and killing of the expressing cell. However, most tumour cell do express tumour-

associated antigens (TAAs). TAAs are usually highly overexpressed in cancer, compared to normal tissues. Another group of TAAs consists of a mutated form of normal antigens, i.e. mucin 1 (MUC1). In healthy tissues, this surface protein in characterised by extensive O-linked glycosylation. In contrast, many malignant tissues express a truncated form of MUC1, whose reduced glycans expose epitopes which are inaccessible on healthy tissues but available targets for antigen-specific therapies (Sørensen *et al.* 2006). The identification several TAAs was the starting point for adoptive T cell therapy of cancer. The major breakthrough for CAR-T cells was the introduction of a costimulatory activation domain in addition to CD3zeta. After the very first generation of CAR-T cells, containing only one single intracellular CD3zeta domain, had shown to be long term persistent in patents, but also not very efficient in eliminating their target cells, a second generation was developed, containing an additional co-stimulatory domain (Gill *et al.* 2016). The most commonly used co-stimulatory domains are CD28 or 4-1BB. This second generation showed a much higher potency compared to the first generation. Incorporation of additional activation domains, mostly CD134, CD137 or OX40 (Zhang *et al.* 2017), amongst others, showed great promise (Tang *et al.* 2016). However, they bear an enhanced risk for cytokine release syndrome (CRS) (Morgan *et al.* 2010; Jin *et al.* 2018). In this case, the release of cytokines by the activated CAR-T cells causes a so called “cytokine storm”. Secreted cytokines stimulate other immune cells, such as T and B cells, macrophages, dendritic and NK cells or monocytes, to produce even more cytokines. This feedback loop results in an overreaction of the immune system (Fitzgerald *et al.* 2017). CRS can also be triggered due to large apoptosis of tumour cells (Dranoff 2004). Interleukin-6 (IL-6) has been shown to be a key mediator for CRS. Therefore, Tocilizumab, an anti-IL-6 monoclonal antibody, has was approved by the FDA to treat CRS, also in CAR-T cell therapy (Le *et al.* 2018). Another approach aiming to enhance efficacy of CAR-T cells is the modification of the tumour microenvironment. Fourth generation CARs (TRUCKs) (Chmielewski and Abken 2015), CAR T cells with inducible release of a transgenic payload. TRUCKs deliver a cassette for the inducible expression of transgenic cytokines, such as IL-12. Signalling within the CAR activation domains induces activation of the NFAT minimal promoter and IL-12 production. Thereupon IL-12 inhibits recruitment of regulatory T cells which would otherwise suppress activation and killing by cytotoxic CAR-T cells.

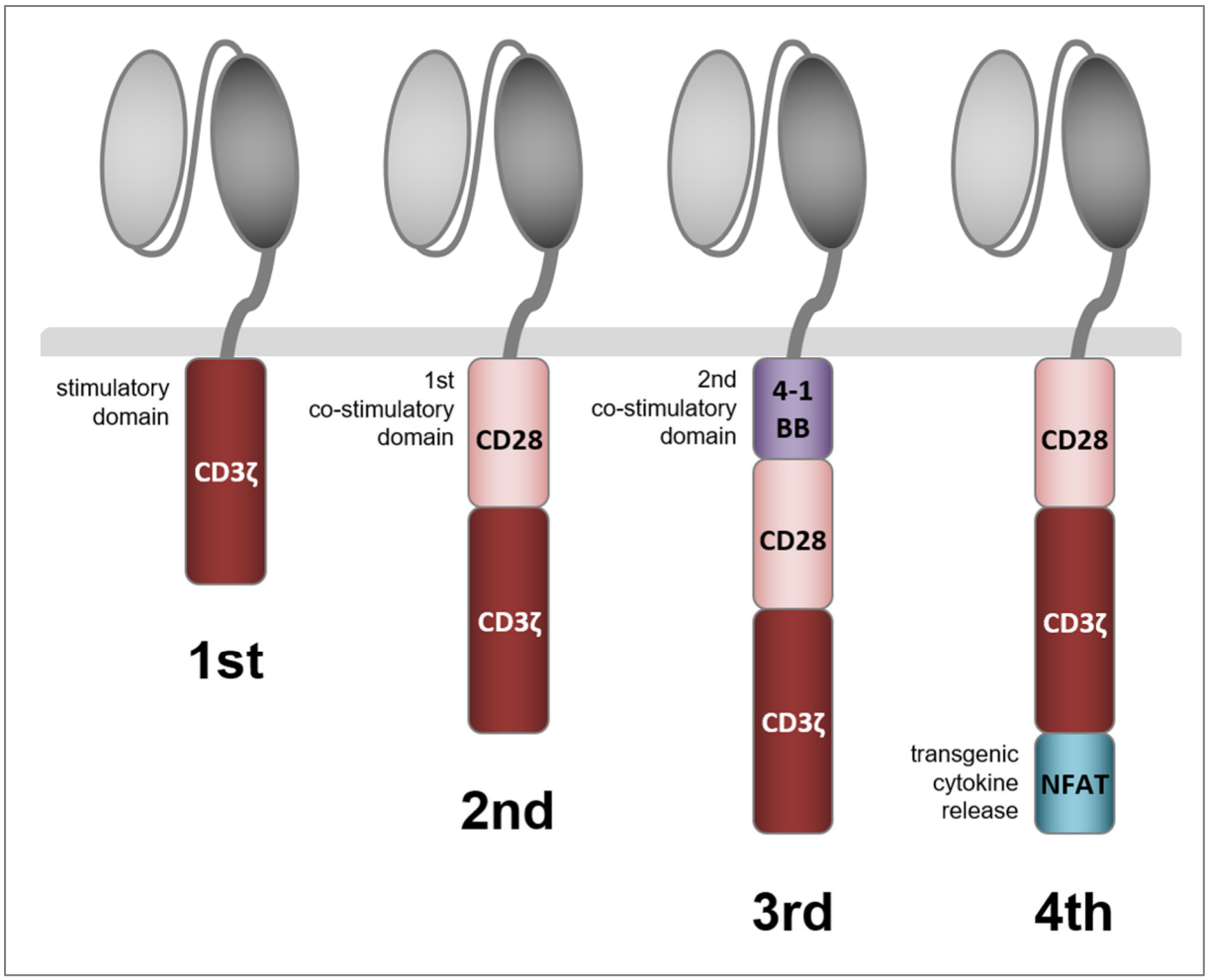

Figure 6: Four Generations of Chimeric Antigen Receptors.

Shown from left to right is the development of the intracellular CAR activation domains. First generation contained only one activation domain whereas for the second generation, a co-stimulatory domain was introduced. Third generation CARs contain two co-stimulatory domains. Fourth generation CARs (TRUCKs) are enabled to also trigger the release of transgenic cytokines.

Until now, CAR-T cell therapy has gained spectacular success in the treatment of haematological malignancies, especially B cells (Hartmann *et al.* 2017; Maude *et al.* 2014). In these assets, B cell lineage marker CD19 was chosen as a target antigen, which is exclusively expressed on B cells, but not on any other cell type within the human body. With this, multiple forms of cancer have been treated, such as acute lymphoblastic leukaemia (ALL), chronic lymphoblastic leukaemia (CLL), and many different forms of Hodgkin's lymphoma. In 2017, the first two CAR-T cell therapies were approved by the FDA. Both are targeting CD19 aiming to treat different types of B cell malignancies. First in class is Novartis' Tisagenlecleucel (commercially Kymriah) has been approved for the treatment of relapsed/refractory B-cell precursor acute lymphoblastic leukaemia (ALL) (Maude *et al.* 2018). Only few months later, FDA also released approval for Yescarta (Axicabtagene ciloleucel) against relapsed/refractory diffuse

large B-cell lymphoma (DLBCL) (Mullard 2017; Sharma *et al.* 2018). Yescarta was initially developed as KTE-C19 by Kite Pharma which was then purchased by Gilead Sciences.

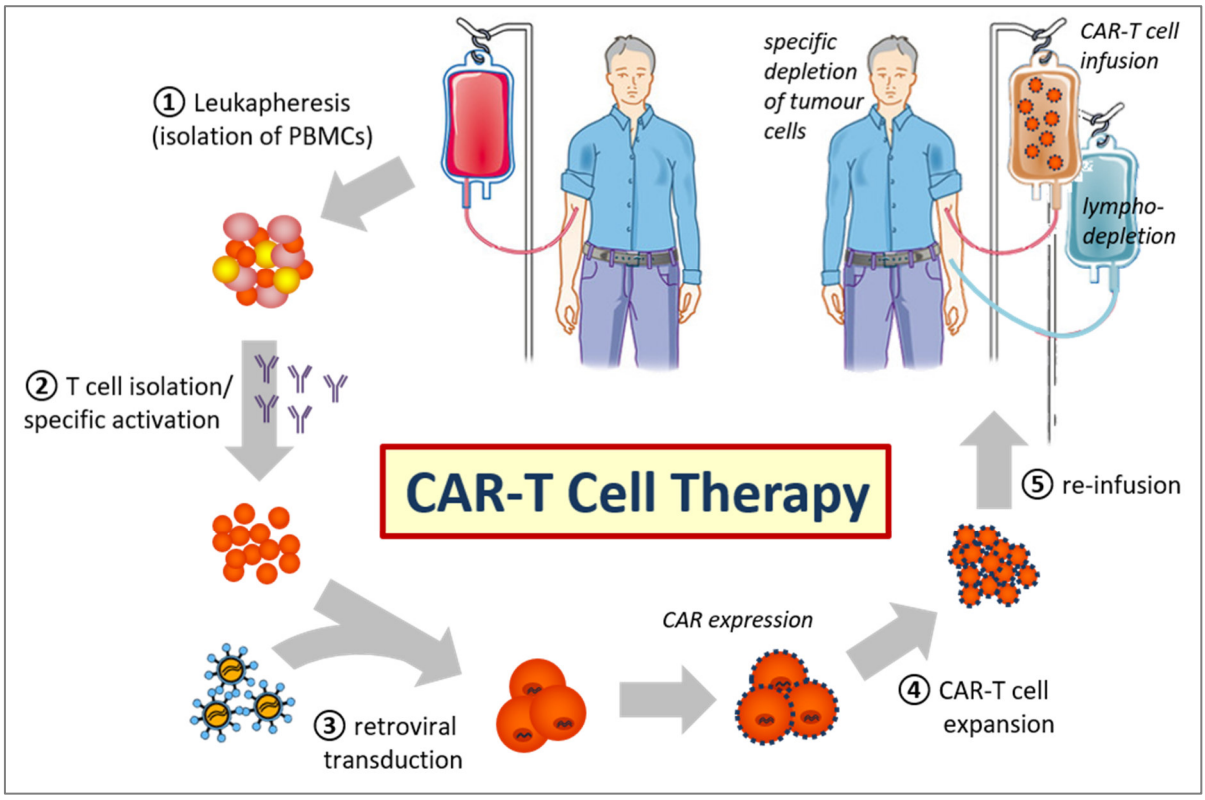

Figure 7: Flow chart of clinical CAR-T cell therapy.

(1) PBMCs are isolated from the patient's blood. **(2)** T cells are specifically activated by anti-CD3 and anti-CD28 antibodies coated on plates or magnetic beads. **(3)** Activated T cells are transduced with retroviral particles, transmitting the CAR genome into the cell. **(4)** CAR-expressing T cells are expanded in vitro using proliferation-inducing cytokines. **(5)** The patient undergoes lymphodepletion to prevent CRS and immune responses against the modified CAR-T cells, which are re-infused into the patient. PBMC = peripheral blood mononuclear cell, CRS = cytokine release syndrome.

CAR-T cells are currently investigated in numerous clinical trials (Figure 8). New targets which are expressed exclusively on the surface of different cancer types are examined either alone, or in combination with a second target in bispecific CARs (Figure 8). CD19 is by far the best evaluated one, followed by BCMA (B Cell Maturation Antigen). Other assets are focusing on the enhancement of CAR-T cells in general for example by knocking out checkpoint inhibitors such as PD-1 (Programmed Cell Death Protein 1). The incorporation of switch technologies is one way of making the CAR-T cell technology safer and more controllable. Figure 8 B shows CAR-T cell clinical trials by indication. The vast majority of trials is carried out in oncology, focusing on liquid tumours, leukaemia and lymphoma. However, with the progressing evolution of the technology, CAR-T cells are now also developed to target solid tumours. Current trials are targeting various malignant diseases from pancreatic over breast and colorectal to lung, renal and prostate tumours (Figure 8 B, dark blue bars). With increasing success, CAR-T cells

have started to be developed for indications outside of oncology (purple bars). Autoimmune diseases such as Lupus and GvHD (Graft versus Host Disease) but also infectious diseases as of CMV (Cytomegalovirus) are potential targets for CAR-T cells. Three trials investigating CAR-T cells for the treatment of HIV infection are listed within the pink bar. After first generation CAR-T cells targeting HIV gp120 have shown long-term persistence but limited efficacy, a trial with second generation CARs is planned targeting conserved domains of gp120 (ChiCTR-OPN-17013068). Another trial has been opened investigating the effect of CAR-T cell therapy on the reconstitution of HIV-specific Immune function (NCT03240328).

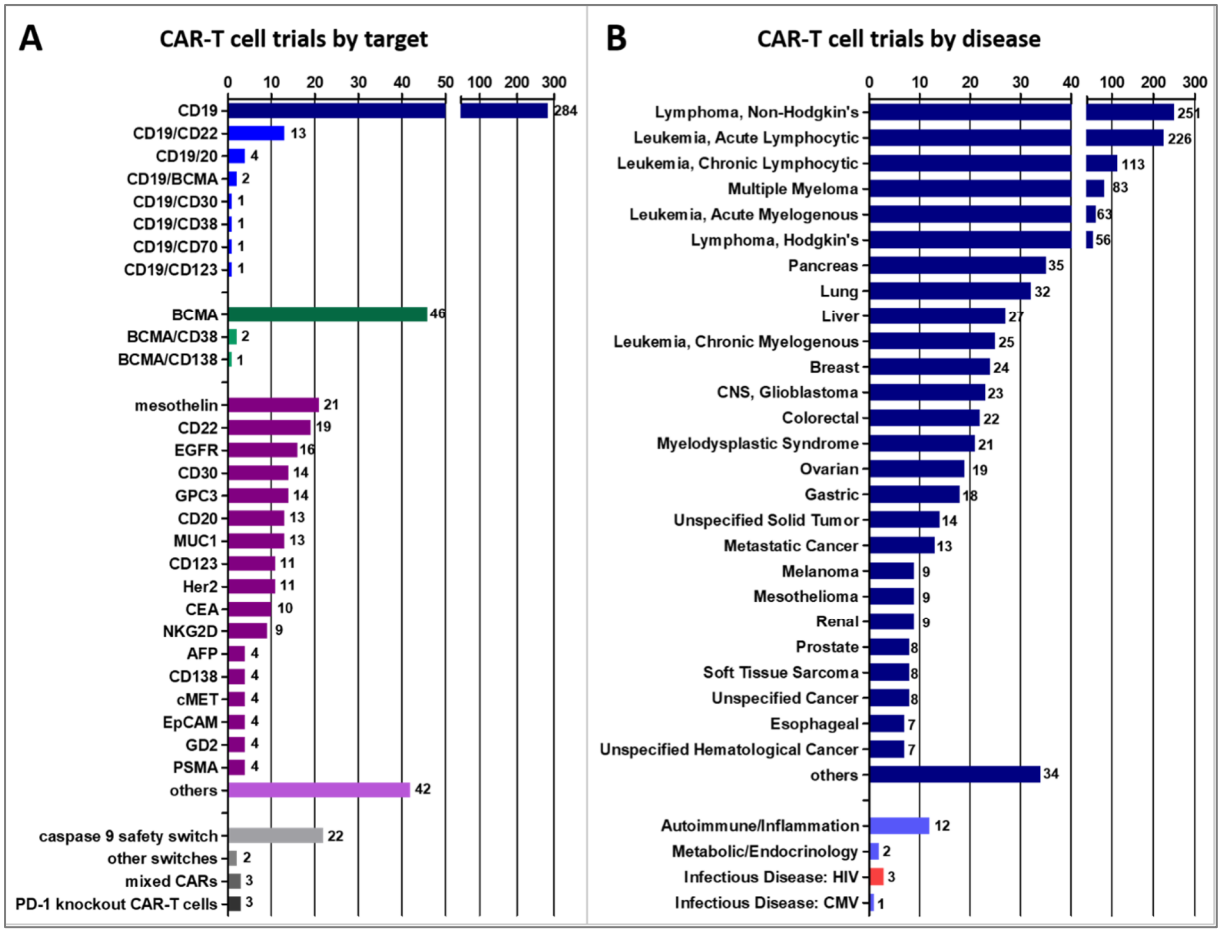

Figure 8: CAR-T Cell Clinical Trials Overview.

Clinical trials investigating CAR-T cells with trial status planned, ongoing or completed. **(A)** Trials sorted by target antigen: Mono- (dark blue) or bispecific (light blue) anti-CD19 CARs; mono- (dark green) or bispecific (light green) anti-BCMA trials; other targets (purple); advanced CAR technologies (grey). **(B)** Trials sorted by disease indication: Oncology (blue); Non-Oncology (purple); HIV (red). Modified from Trialtrove as of October 2018 (Informa PLC 2018).
(BCMA = B Cell Maturation Antigen; EGFR = Epidermal Growth Factor Receptor; GPC = Oncofetal Antigen Glypican; MUC1 = Mucine1; Her2 = Human Epidermal Growth Factor Receptor 2; CEA = Carcinoembryonic Antigen; NKG2D = Natural Killer Group 2D; AFP = Alpha-Fetoprotein; cMET = c-terminal MET Proto-Oncogene; EpCAM = Epithelial Cell Adhesion Molecule; GD2 = Ganglioside 2D; PSMA = Prostate-Specific Membrane Antigen; PD-1 = Programmed Cell Death Protein 1; CNS = Central Nervous System; HIV = Human Immunodeficiency Virus; CMV = Cytomegalovirus)

Despite the great survival rates of patients suffering from B cell malignancies, a whole lot of development is still required in order to make CAR-T cell therapy applicable to a broader range of patients with various other types of cancer (Maude 2017). To be able to also increase efficacy and decrease risks of side effects, huge effort is made within academic and industrial research (Pang *et al.* 2018; Jindal *et al.* 2018; Yee 2018). After the very first generation of CAR-T cells, containing only one single intracellular CD3zeta domain, had shown to be long term persistent in patents, but also not very efficient in eliminating their target cells, a second generation was developed, containing an additional co-stimulatory domain (Gill *et al.* 2016). The most commonly used co-stimulatory domains are CD28 or 4-1BB. This second generation showed a much higher potency compared to the first generation. Incorporation of additional activation domains, mostly CD134, CD137 or OX40 (Zhang *et al.* 2017), amongst others, showed great promise (Tang *et al.* 2016). However, they bear an enhanced risk for cytokine release syndrome (CRS) (Morgan *et al.* 2010; Jin *et al.* 2018). In this case, the release of cytokines by the activated CAR-T cells causes a so called “cytokine storm”. Secreted cytokines stimulate other immune cells, such as T and B cells, macrophages, dendritic and NK cells or monocytes, to produce even more cytokines. This feedback loop results in an overreaction of the immune system (Fitzgerald *et al.* 2017). CRS can also be triggered due to large apoptosis of tumour cells (Dranoff 2004). Interleukin-6 (IL-6) has been shown to be a key mediator for CRS. Therefore, Tocilizumab, an anti-IL-6 monoclonal antibody, has was approved by the FDA to treat CRS, also in CAR-T cell therapy (Le *et al.* 2018). Another approach aiming to enhance efficacy of CAR-T cells is the modification of the tumour microenvironment. Fourth generation CARs (TRUCKs) (Chmielewski and Abken 2015), CAR T cells with inducible release of a transgenic payload. TRUCKs deliver a cassette for the inducible expression of transgenic cytokines, such as IL-12. Signalling within the CAR activation domains induces activation of the NFAT minimal promoter and IL-12 production. Thereupon IL-12 inhibits recruitment of regulatory T cells which would otherwise suppress activation and killing by cytotoxic CAR-T cells.

1.2.2 Chimeric antigen receptors for HIV therapy

The approach to use CAR-T cells as a treatment for HIV shifted out of the scientific focus (Riddell *et al.* 1996; Roberts *et al.* 1994; Wagner 2018). This was mostly due to the limited success of anti-HIV CAR-T cells in early years which produced low efficiency due to poor persistence in the long-term (Scholler *et al.* 2012). However, in these cases, first generation CARs containing only the intracellular CD3zeta domain were used, which was not sufficient for full T cell activation. Driven by the increasing success of new generation TCR and CAR-T cells against cancer, new hope was raised to be able to carry this forward into HIV research (Wagner 2018).

Transferring the principle of CAR-T cells from treatment of cancer back to HIV became evident. Haematological malignancies such as leukaemia or lymphoma are diseases of leukocytes caused by mutations of their DNA. These mutations lead to significant changes of the cell metabolism, disrupting their native functions within the human body. In addition, uncontrolled cell proliferation impairs the supply of healthy cells with essential metabolites and oxygen which results in failure of vital organs. Chronic HIV-infection shows many similarities. Disease´s origin is the integration of reverse-transcribed viral DNA into the human immune cell. Loss of function or even death of infected cells also leads to organ failure. In contrast to cancer, HIV spreads by replication of viral particles which are transferred intercellular, rather than proliferation of the cell itself. To be treated by CAR-T cells, any disease requires one main characteristic: a unique target which is sufficiently expressed on the surface of all affected cells but nowhere on healthy cells or tissues. The most successful CAR-T cell target so far has been CD19. It is expressed exclusively on B cells which can be fully depleted followed by sufficient recovery after termination of the therapy. Other potential targets for CAR-T cells are currently investigated in clinical trials. These tumour antigens which are highly upregulated in cancer cells are e.g. CD20, CD30, or BCMA in haematological malignancies as well as ErbB2/Her2, VEGFR, CAIX and CEA in solid tumours (Hartmann *et al.* 2017).

In order to achieve a functional cure, meaning the immunological control of HIV infection, CAR-T cells must be able to overcome HIV immune escape and be effective against a broad variety of HIV strains. In addition, anti-HIV CAR-T cells must not be immunogenic to guarantee long-term persistence.

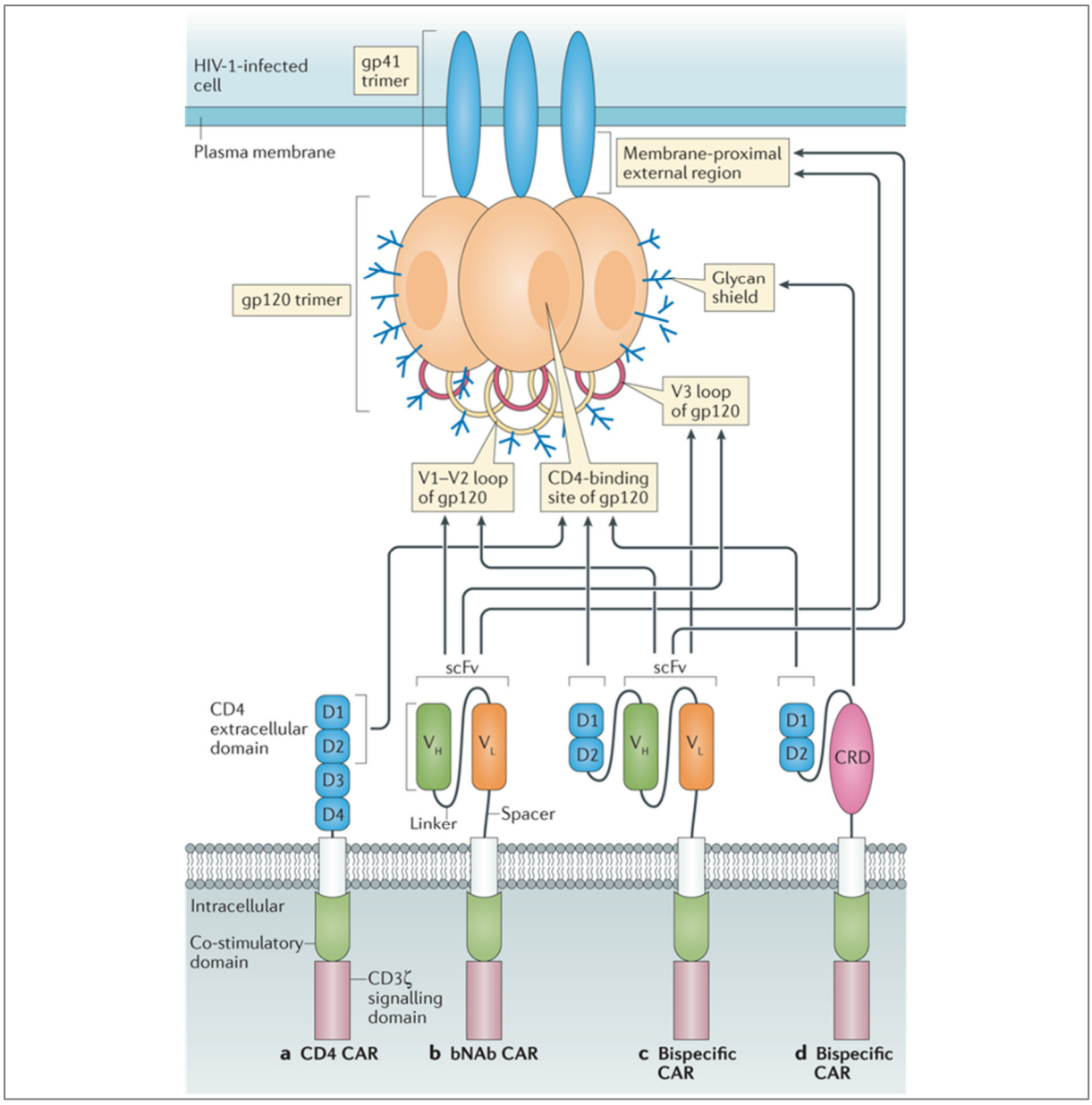

Figure 9: CD4-based CARs against HIV.

Extracellular CAR binding domains specifically target different epitopes of the HIV envelope protein (env). **(a)** Out of the four extra-cellular CD4 domains, D1 and D2 are crucial for binding of env gp120. **(b)** CARs using single-chain variable fragments (scFvs) derived from broadly neutralising anti-bodies (bNAbs) bind env via CD4-binding site or second variable loop (V2). **(c, d)** Bispecific anti-HIV CARs bind alternative regions of env or the carbohydrate recognition domain (CRD) of a C- type lectin to glycan motifs. VH = variable heavy chain; VL = variable light chain. Figure: Maldini *et al.* 2018

CARs against HIV infected cells so far have been targeted against viral proteins, in particular glycoprotein (gp) 120. Gp120 is anchored in the viral matrix and the lipid membrane of the host cell, where it is expressed on the cell surface. Aligned with observations in cancer therapy, incorporation of a second costimulatory domain into the CAR has also shown significant increase of potency compared to first generation HIV-specific CAR-T cells (Leibman *et al.* 2017).

An overview of new generations of anti-HIV CAR-T cells has been reviewed by Maldini *et al.* 2018. The majority of HIV-specific CARs is based on the binding and entry of HIV env protein gp120 via CD4 receptor on the target cell (Carrillo *et al.* 2017; Ghanem *et al.* 2018; Kuhlmann *et al.* 2018). The most “native” example is a CAR binding domain consisting of full-length CD4 with domains D1-D4, which targets the CD4-bidig site of gp120. This binding domain is linked to CD3zeta by a 4-1BB or CD28 costimulatory domain (Leibman *et al.* 2017). In a humanised mouse model, these improved CAR-T cells showed around 50-fold decrease of viral RNA compared to the clinical CAR without a costimulatory domain. Further promising results have been achieved targeting gp120 with CAR-T cells derived from broadly neutralizing antibodies (bNAbs) (Ali *et al.* 2016; Carrillo *et al.* 2017). Hereby, highly conserved domains of gp120 are targeted, which decreases the chance of immune escape due to the high mutation rate (Woo *et al.* 2010). Favourite targets of extracellular scFvs derived from bNAbs are the variable loops V1-V3 expressed on the gp120 subunits (Huang *et al.* 2016) or an extracellular epitope on gp41 (Nelson *et al.* 2007). Another advancement was the development of bispecific CARs, carrying two coupled extracellular binding domains (Patel *et al.* 2000). Therefore, CD4 domains D1+D2 recognise the CD4 binding site of gp120, followed by a scFv domain specific for a variable loop of gp41 (Liu *et al.* 2015). Bispecific CARs enable T cell activation upon binding of either one or the other binding domain (Carrillo *et al.* 2017). Thus, the alternative target increases the chance of recognition by the T cell. Another bispecific CAR used carbohydrate recognition domain (CRD) of human lectin to target the highly conserved glycan shield on gp120 (Ghanem *et al.* 2018). Because CRD is also capable of binding endogenous cell components as expressed by healthy cells (Ip *et al.* 2009), the possibility of on-target/off tissue toxicity is increased.

Success of HIV specific CAR-T cells targeting vial env proteins is increasing rapidly (Kuhlmann *et al.* 2018). However, epitopes of HIV env protein are only present on the cell surface as long as the provirus is actively transcribed and mRNA is translated into peptides. This is mainly the case when the host cell is activated. As soon as the virus enters a latent state, viral proteins are no longer translated and exposed. However, the majority of patients with chronic HIV infection receives cART to suppress viral replication and spreading, as described previously (Section 1.1.4). By targeting epitopes within env, only HIV-expressing cells are recognized and depleted, leaving the latent viral reservoir intact (Huyghe *et al.* 2017; Lam and Bollard 2013). To be able to target latently infected cells, an exclusive but at the same time omnipresent cellular marker is required. Despite intensive research during the last decades, a marker expressed by latently infected cells could still not be identified (Pillai and Deeks 2017).

As described in Section 1.1.5, the existence of such a HIV latency maker is highly unlikely, because it is the strategy and concept of a latent provirus to remain silent and undetectable for the entire immune system. CAR-T cells targeting viral proteins are therefore only capable

of a “functional cure” which controls viremia without additional treatment, but not a “sterilising cure” which eradicates all forms of HIV from the patient.

1.2.3 CD4–specific DARPins as CAR binding domains

To be able to direct T cells against HIV infected cells including the latent reservoir, it is not sufficient to just target epitopes of viral proteins. Nevertheless, binding of viral gp120 to cellular CD4 is the key event during HIV infection and remains constant over all replication cycles. Viral proteins are subject to high mutation rates and once the virus enters a latent stage, a cell can no longer be identified as infected. However, every potentially HIV infected cell expresses CD4, since this is its exclusive entry receptor (Schnittman *et al.* 1990). CD4 is therefore the lowest common denominator of all HIV infected or potentially infected cells. Specific depletion of $CD4^+$ cells would therefore eliminate actively HIV producing, as well as latently HIV infected cells. CD4-depleting antibodies have shown efficacy in chimpanzees (Jonker *et al.* 1993). Zanolimumab, a monoclonal antibody against human CD4 has been used for treatment cutaneous and nodal T cell lymphomas (Rider *et al.* 2007). Zanolimumab inhibits early TCR signalling and activation of CD4-associated tyrosine-kinase p56 Lck, leading to apoptosis of CD4 T cells. This therapy has been found safe and well-tolerated by patients with inflammatory diseases, such as rheumatoid arthritis (Skov *et al.* 2003). One step further is the transfer of the CD4-specific binding domain to a CAR. Also CAR-T cells targeting CD4 have shown efficacy and safety in preclinical models, aiming to defeat T cell malignancies (Pinz *et al.* 2016). In this project, the approach of specific CD4 T cell depletion was transferred to HIV infection. For this approach, many complex processes need to be understood: activation of CAR-T cells, CD4 target cells and other immune cells, effects of cytokine release on immune response, viral replication and reversing of HIV latency, as well as CAR-T cell persistence and proliferation and tolerance of absence of CD4 T cells and their recovery. These effects and their correlations must be investigated in a suitable model. So far, the only fully immunocompetent animal model for HIV are non-human primates (nhps) (Policicchio *et al.* 2016), since they are the natural host of SIV. Apart from nhps and humans, no other species became susceptible to SIV/HIV or a related virus strain. To be able to show efficacy of CD4 specific CAR-T cells against HIV infection *in vivo*, we chose an extracellular binding domain which has cross-reactivity towards human and nhps (Schweizer *et al.* 2008).

Instead of the commonly used scFv (single chain variable fragment) antibody for binding, we used a well-characterized anti-CD4-DARPin (designed ankyrin repeat protein) (Schweizer *et al.* 2008; Zhou *et al.* 2015). DARPins are derived from ankyrin repeat proteins which are among the most common structural binding motifs in nature and can be used to specifically bind a variety of defined targets.

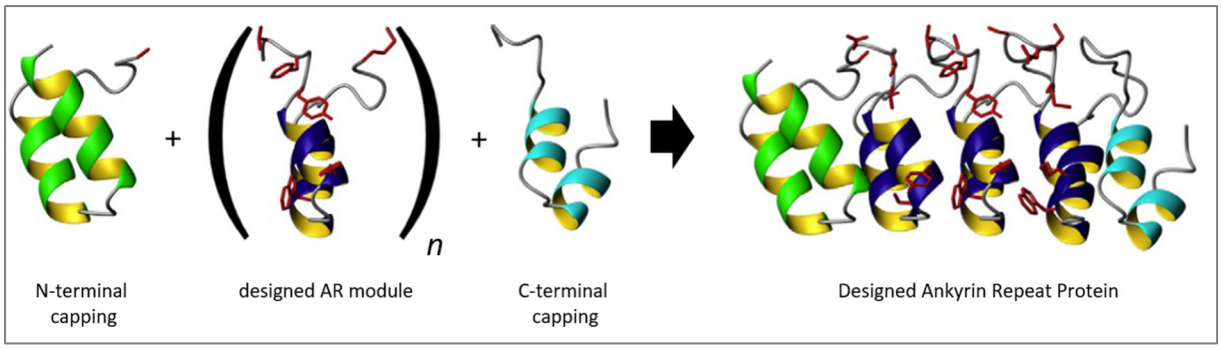

Figure 10: DARPin structure.

DARPins consist of designed Ankyrin modules with several repeats (AR), flanked by N- and C-terminal capping. Modified from Tamaskovic *et al.* 2012.

DARPins are isolated by phage or ribosomal display from libraries, to select the best candidate to bind a given target protein. DARPins were previously used to target antigens that are expressed at even very low levels (Tamaskovic *et al.* 2012) in a very efficient way for use in gene therapy (Friedrich *et al.* 2013; Munch *et al.* 2011). DARPINs are small molecules and exhibit an extraordinary high thermodynamic stability (Wetzel *et al.* 2008), affinity and specificity (Binz *et al.* 2003); in contrast to antibodies DARPins do not require pairing or linking of two polypeptide chains to form functional binding domains. First clinical trials using DARPins are aiming at the treatment of macular degeneration (Ferrara *et al.* 2003; Campochiaro *et al.* 2013; NCT02194426; NCT02462928; NCT02462486) . Thus, DARPins are a promising alternative to scFv antibodies as binding domains for CAR-T cells. DARPins have already proven to be a specific and reliable binding domain for CAR-T cells, targeting tumour-antigen Her2 (Hammill *et al.* 2015; Siegler *et al.* 2017).

1.3 Objective

The overall objective of this thesis was to investigate the proof-of-concept of anti-CD4-DARPin CAR-T cells, and whether targeting a cellular protein could be a potential strategy to achieve eradication of HIV-infected cells, including the latent reservoir.

Therefore, the anti-CD30 binding domain of a well-established 2nd generation CAR, containing a CD3/CD28 activation domain, should be replaced by an anti-CD4-DARPin. Once this anti-CD4-DARPin CAR could be stably expressed on the surface of primary T cells, its functionality needed to be tested. For this purpose, T cell activation should be determined by monitoring secretion of T cell specific cytokines in the presence of target antigen. At the same time, cytotoxicity of anti-CD4-DARPin CAR-T cells towards antigen-positive target cells had to be determined in a suitable killing assay. Specificity towards the target antigen is a major demand for CARs to prevent off-target toxicity. Therefore, CAR-T cell activation and cytotoxicity had to also be tested towards target-negative cells. In order to achieve a sterilising cure from HIV, it is essential to also eliminate the entire reservoir of latently infected cells. However, towards the end of a potential therapy, CD4 target cells would be a very rare population in the patient. One arm of the cytotoxicity assays should therefore examine how efficiently a very rare target population can be depleted. Subsequently, efficacy of anti-CD4-DARPin CAR-T cells needed to be tested in a HIV-latency model. The main questions to be addressed were firstly, whether depletion of CD4 T cells would reduce or even fully anticipate reactivation of latent HIV provirus. Secondly, it had to be tested if, besides the latent reservoir, activated HIV-expressing cells are depleted as well. CAR-T cell therapy uses autologous T cells which are isolated from the of patient's blood. After transduction, confirmed CAR expression and expansion *in vitro*, CAR-T cells are reinfused back into the patient to deplete their target cell population. In addition to immortalised cell lines, anti-CD4-DARPin CAR-T cells therefore had to also be challenged in an autologous system, in which primary CAR-T and CD4-positive target cells are derived from the same donor. In order to fully understand the effects along with potential risks and benefits of anti-CD4-DARPin CAR-T cells, testing in an immunocompetent animal model is required. In the case of HIV, SIV-infected non-human primates are the most suitable model. Hence, an equivalent nhp anti-CD4-DARPin CAR had to be designed, in which the intracellular CAR activation domains would be capable to induce activation of nhp T cells. Should these nhp-based anti-CD4-DARPin CARs show similar efficacy in an autologous *ex vivo* setting, they could serve as a platform to test the approach in an immunocompetent nhp model.

In summary, this thesis aimed to combine three promising approaches within one concept:
(1) The potency of a second-generation CAR to drive efficient T cell activation (2) in combination with a DARPin as a specific, stable and efficient targeting domain and (3) CD4 as a cellular target to deplete potentially HIV-positive cells, including those in a latent stage.

2 Material and Methods

2.1 Material

2.1.1 Consumables

Table 2: Consumables

Name	Cat. no.	Source of Supply
Amersham™ Hybond™ P 0.45 µm-PVDF-membrane	15259894	GE Healthcare, Little Chalfont, UK
Cellometer Disposable Counting Chambers	CHT4-PD100-002	Nexelom
CellTrace™ CFSE Cell Proliferation Kit	C34554	Thermo Fisher Scientific, Waltham/MA, USA
CellTrace™ Violet Cell Proliferation Kit	C34557	Thermo Fisher Scientific, Waltham/MA, USA
Cryo freezing container	5100-0001	Nalgene
Cryo-tubes (2 ml)	V3135	Greiner Bio-One
Dimethyl sulfoxide	CAS 67-68-5	Sigma-Aldrich, Munich, Germany
EZ-VISION® THREE, DNA dye as loading buffer	N313	Amresco, Solon/OH, USA
Fixable Viability Dye eFluor™ 780	65-0865-14	eBioscience™
High performance chemiluminescent films	28906844	GE Healthcare
Micro centrifuge tubes (1.5 ml)	30120086	Eppendorf
Monkey IFN-γ ELISA development kit (HRP)	3421M-1H	Mabtech, Stockholm, Sweden
NucleoBond® Xtra Maxi kit	740414	MACHEREY-NAGEL, Düren, Germany
NucleoSpin® Plasmid kit	740490	MACHEREY-NAGEL, Düren, Germany
NucleoSpin® Gel and PCR Clean-up	740609	MACHEREY-NAGEL, Düren, Germany
NuPAGE™ Novex™ 4-12% Bis-Tris Protein Gels, 1.5 mm, 15-well	NP0336BOX	Thermo Fisher Scientific, Waltham/MA, USA
PCR-tubes, thin walled, 200 µl	30124332	Eppendorf
Pipette tips, filtered (10 µl, 100 µl, 300 µl, 1000 µl)	-	Nerbe Plus
Protein marker XXL DeLuxe	310007	GeneOn GmbH, Ludwigshafen, Germany
Serological pipettes (2 ml, 5 ml, 10 ml, 25 ml)	-	Greiner Bio-One
Syringe filters, Minisart PTFE (0.45 µm, 0.2 µm)	17576K, 17573K	Satorius
Tissue culture flask (T25, T75, T175)	-	Greiner Bio-One
Tissue culture plates, 6-well	140675	Nunc, Thermo-Fisher Scientific
Tissue culture plates, 12-, 24-well	CLS3524	Corning, Sigma
Tissue culture plates (96-well-U-bottom)	163320	Nunc, Thermo-Fisher Scientific
Tissue culture plates (96-well-V-bottom)	651101	Greiner Bio-One

Name	Cat. no.	Source of Supply
Tissue, Cell Tork	530178	Tork
Tubes, sterile (15 ml, 50 ml)	188271, 227261	Greiner Bio-One
VACUETTE® TUBE 9 ml 9NC Coagulation sodium citrate	455322	Greiner Bio-One
VWR® Vacuum Filtration 0.45 µm PES	97066-208	VWR Darmstadt, Germany
Zero Blunt™ TOPO™ PCR Cloning Kit	450245	Invitrogen

Table 3: Reagents

Name	Cat. no.	Source of Supply
Acetic acid, CH_3COOH	3738	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
2-Log DNA Ladder (0.1-10.0 kb)	N3200	New England Biolabs, Ipswich/MA, USA
2-Propanol, C_3H_8O	6752	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Amersham ECL Prime Western Blotting Detection Reagent	RPN2232	GE Healthcare, Little Chalfont, UK
Ammonium chloride 0.87%	A9434	Sigma-Aldrich, Munich, Germany
Ampicillin , D-(−)-α-Aminobenzylpenicillin	K029	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Biozym LE Agarose	840004	Biozym Scientific GmbH, Hessisch Oldendorf, Germany
Bradford Reagent	B6916	Sigma-Aldrich, Munich, Germany
CellTrace™ CFSE Cell Proliferation Kit	C34554	Thermo Fisher Scientific, Waltham/MA, USA
CellTrace™ Violet Cell Proliferation Kit	C34557	Thermo Fisher Scientific, Waltham/MA, USA
Ethanol, C_2H_6O	5054	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
EZ-VISION® THREE, DNA dye as loading buffer	N313	Amresco, Solon/OH, USA
Histopaque®-1077	10771	Sigma-Aldrich, Munich, Germany
L-Glutamine	F7513	Sigma-Aldrich, Munich, Germany
Methanol, CH_3OH	T909	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Monkey IFN-γ ELISA development kit (HRP)	3421M-1H	Mabtech, Stockholm, Sweden
NucleoBond® Xtra Maxi kit	740414	MACHEREY-NAGEL, Düren, Germany
NucleoSpin® Plasmid kit	740490	MACHEREY-NAGEL, Düren, Germany
NucleoSpin® Gel and PCR Clean-up	740609	MACHEREY-NAGEL, Düren, Germany
NuPAGE® Antioxidant	NP0005	Thermo Fisher Scientific, Waltham/MA, USA
NuPAGE® Sample Reducing Agent (10X)	NP0004	Thermo Fisher Scientific, Waltham/MA, USA
NuPAGE™ Novex™ 4-12% Bis-Tris Protein Gels, 1.5 mm, 15-well	NP0336BOX	Thermo Fisher Scientific, Waltham/MA, USA
Paraformaldehyde (PFA)	P6148	Sigma-Aldrich, Munich, Germany
Penicillin-Streptomycin	17-602E	Lonza, Basel, Switzerland
Polyethylenimine (PEI)	408727	Sigma-Aldrich, Munich, Germany

Name	Cat. no.	Source of Supply
poly-L-lysine (PLL)	P6282	Sigma-Aldrich, Munich, Germany
Powdered milk	T145	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Proleukin® S	PZN 02238131	Novartis Pharma, Basel, Switzerland
Prostatin	P0077	Sigma-Aldrich, Munich, Germany
Protamine sulfate	P4020	Sigma-Aldrich, Munich, Germany
Protein marker XXL DeLuxe	310007	GeneOn GmbH, Ludwigshafen, Germany
SAHA	SML0061	Sigma-Aldrich, Munich, Germany
Sodium azide (NaN_3)	106688	Merck KGaA, Darmstadt, Germany
Sulfuric acid (95-97% H_2SO_4)	100731	Merck Millipore, Darmstadt, Germany
TMB/E (Horseradish Peroxidase Substrate)	ES001	Merck Millipore, Darmstadt, Germany
Trypan blue, cell viability	CN76.1	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Tween® 20	9127	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Acetic acid, CH_3COOH	3738	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
2-Propanol, C_3H_8O	6752	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Amersham ECL Prime Western Blotting Detection Reagent	RPN2232	GE Healthcare, Little Chalfont, UK
Ammonium chloride 0.87%	A9434	Sigma-Aldrich, Munich, Germany
Biozym LE Agarose	840004	Biozym Scientific GmbH, Hessisch Oldendorf, Germany
Ethanol, C_2H_6O	5054	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Methanol, CH_3OH	T909	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
NuPAGE® Antioxidant	NP0005	Thermo Fisher Scientific, Waltham/MA, USA
NuPAGE® Sample Reducing Agent (10X)	NP0004	Thermo Fisher Scientific, Waltham/MA, USA
Paraformaldehyde (PFA)	P6148	Sigma-Aldrich, Munich, Germany
Polyethylenimine (PEI)	408727	Sigma-Aldrich, Munich, Germany
poly-L-lysine (PLL)	P-6282	Sigma-Aldrich, Munich, Germany
Powdered milk	T145	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Protamine sulfate	P4020	Sigma-Aldrich, Munich, Germany
Sodium azide (NaN_3)	106688	Merck KGaA, Darmstadt, Germany
Sulfuric acid (95-97% H_2SO_4)	100731	Merck Millipore, Darmstadt, Germany
TMB/E (Horseradish Peroxidase Substrate)	ES001	Merck Millipore, Darmstadt, Germany
Trypan blue, cell viability dye	CN76.1	Carl Roth GmbH + Co. KG, Karlsruhe, Germany
Tween® 20	9127	Carl Roth GmbH + Co. KG, Karlsruhe, Germany

Table 4: Antibodies

Name	Cat. no.	Source of Supply
anti-human CD4-PE, clone SK3	347327	Beckton Dickinson, Heidelberg, Germany
CD28 pure, clone 15E8	130-093-375	Miltenyi Biotech, Bergisch Gladbach, Germany
CD30-PE, human, clone Ki-2	130-081-401	Miltenyi Biotech, Bergisch Gladbach, Germany
CD3-PerCP-Vio700, clone 10D12	130-104-204	Miltenyi Biotech, Bergisch Gladbach, Germany
CD4 MicroBeads, human	130-045-101	Miltenyi Biotech, Bergisch Gladbach, Germany
CD8 (Human), APC, clone B9.11	IM2469	Beckman Coulter GmbH, Brea/CA, USA
FITC Mouse Anti-Human CD4, clone RPA-T4	555346	BD Pharmingen™, Heidelberg, Germany
FITC Mouse Anti-Human CD4, clone L200	550628	BD Pharmingen™, Heidelberg, Germany
Goat F(ab')$_2$ Anti-Human IgG, Mouse ads-PE	2043-09	Southern Biotech, Birmingham/AL, USA
PE Mouse IgG1, κ Isotype Control Clone MOPC-21	559320	BD Pharmingen™, Heidelberg, Germany
PerCP Mouse Anti-Human CD3, clone SP34.2	552851	BD Pharmingen™, Heidelberg, Germany
Primary antibody: antiserum against Rauscher Murine Leukaemia Virus p30	NCI HD 539, ATCC® VR1564ASGt™	American Type Culture Collection (ATCC), Manassas/VA, USA
Purified anti-human CD3, clone OKT-3	23333A	Janssen-Cilag, Neuss
Purified anti-human CD3, clone SP34.2	551916	BD Pharmingen™, Heidelberg, Germany
Secondary antibody: Peroxidase AffiniPure Donkey Anti-Goat IgG (H+L)	705-035-147	Jackson ImmunoResearch Laboratories, West Grove/PA, USA
APC Mouse Anti-Human CD25	555434	BD Pharmingen™, Heidelberg, Germany
APC Mouse Anti-Human CD69	555533	BD Pharmingen™, Heidelberg, Germany

Table 5: Buffers

Name	Cat. no.	Source of Supply
Blocking solution	1 x TBS-T 5 % powdered milk 20 % horse serum	Self mix
FACS wash buffer (MACS)	400 g NaCl 10 g KCl 10 g KH_2PO_4 57.5 g Na_2HPO_4 ad ddH_2O 50 l, pH 7.1	PEI department for media and solutions
MOPS running buffer (20x)	2092 g MOPS (3-(N-morpholino)propanesulfonic acid) (1 M) 1212 g Tris(hydroxymethyl)-aminomethan (1 M) 200 g SDS (69.3 mM) 60 g EDTA (20.5 mM) ad ddH_2O 10 l	PEI department for media and solutions
NuPAGE® LDS Sample Buffer (4x)	NP0007	Thermo Fisher Scientific, Waltham/MA, USA
NuPAGE® Transfer Buffer (1x)	25 ml NuPAGE® Transfer Buffer (20x) 0.5 ml NuPAGE® Antioxidant 50 ml Methanol ad ddH_2O 500 ml	Thermo Fisher Scientific, Waltham/MA, USA
NuPAGE® Transfer Buffer (20x)	NP0007	Thermo Fisher Scientific, Waltham/MA, USA
PBS + 0.05% Tween 20	PBS + 0.05% Tween 20	PEI department for media and solutions
Phosphate buffered saline (PBS)	800 g NaCl 20 g KCl 20 g KH_2PO_4 115 g Na_2HPO_4 add ddH_2O 100 l, pH 7.1	PEI department for media and solutions
TAE buffer (20x)	9680 g Tris(hydroxymethyl)-aminomethan (TRIS) 2284 ml glacial acetic acid 4 l EDTA (0.5 M, pH 8.0) ad ddH_2O 50 l	PEI department for media and solutions
Tris-buffered saline 0.1 % Tween (TBS-T)	605.5 g Tris(hydroxymethyl)-aminomethan (50 mM) 876.6 g NaCl (150 mM) 100 ml Tween 20 ad ddH_2O 100 l, pH 7.4	PEI department for media and solutions
Trypsin-EDTA (0.05%Trypsin)	5 g Trypsin 250 2g Titriplex III ad PBS 10 l	PEI department for media and solutions

Table 6: Media and sera

Name	Cat. no.	Source of Supply
Dulbecco's Modified Eagle Medium (DMEM)	L0106	BioWest SAS, Nuaillé, France
Bovine Serum Albumin (BSA)	A9418	Sigma-Aldrich, Munich, Germany
Donor horse serum	S9133, lot 0096T	Biochrom AG, Berlin, Germany
Fetal Bovine Serum (FBS)	F7524, lot 103M3395	Sigma-Aldrich, Munich, Germany
LB medium	10 g tryptone from Casein 5 g yeast extract 10 g NaCl ad 1 l ddH_2O, pH 7.5	PEI department for media and solutions
LB-Amp agar plates	40 g LB-Agar 0.1 mg/ml Ampicillin ad 1 l ddH_2O	PEI department for media and solutions
RPMI 1640	L0501	BioWest SAS, Nuaillé, France
S.O.C. medium	26.6 g SOB-medium 20 mM glucose ad 1 l ddH_2O	PEI department for media and solutions

2.1.2 DNA plasmids and primers

Table 7: Plasmids

Name	Description
pHIT60	gag-pol
pcDNA_GaLVwt (pCOLT)	Env of gibbon ape leukaemia virus
pcDNA3.1(-)	Subcloning non-expression plasmid
#1138, pBullet-Lk-HRS3scFv-DFc-CD28DLck-CD3zeta	Original MLV-based CAR expression plasmid (Hombach *et al.* 1998), with kind permission from Hinrich Abken (Universitätsklinikum Köln)
pBullet-Igk-HA-HRS3scFv-DFc-huCD28DLck-CD3zeta	CAR expression plasmid derived from #1138 with inserted HA-tag and restriction sites
pCG-Hnse-DARPin-d18-CD4_57.2.	Subcloning of DARPin-d18-CD4_57.2, with kind permission from Alexandra Trkola University of Zürich)
pBullet-Lk-DARPin-d18-CD4_57.2-Myc-DFc-huCD28DLck-CD3zeta	CAR expression plasmid based on #1138 with inserted Myc-tag and anti-CD4-DARPin binding domain
pQE-HisHA-CD8.H2C3	Subcloning of control DARPin H2C3
pBullet-Igk-HA-DARPin_CD8.H2C3-DFc-huCD28DLck-CD3zeta	CAR expression plasmid based on #1138 with inserted HA-tag and unspecific H2C3-DARPin binding domain
13ABXFBP_1434780_RhCAR1	Subcloning of rhesus CD3/CD28wt CAR activation domain
15AAUQSP_1661810_AGM_CD28CD3zeta	Subcloning of AGM CD3/CD28wt CAR activation domain

Table 8: Primer

Short term	Name	Sequence (5' → 3')
LP30	XhoI-SbfI-CD3zeta-rev	CTCGAGCCTGCAGGTTAGCGAGGGGGCAGGGCCTG
LP55	BamHI-NcoI-Lkappa-SfiI-DARPin-fwd	GGATCCATGGATTTTCAGGTGCAGATTTTCAGCTTCCTGCTAATCAGTGCCTCAGTCATAATGTCTCAGGCCCAGCCGGCCGACCTGGGTAAGAAACTGC
LP56	DARPin-NotI-Myc-rev	CCTCTTCTGAGATGAGTTTTTGTTCGGCGGCCGCATTAAGCTTTTGCAGGATTTCAGC
LP57	Myc-PacI-IgG-fwd	CTCATCTCAGAAGAGGATCTGTTAATTAACGCCGAGCCCAAA
LP60	CD28 Lck binding motif mutation 3x Pro to Ala fwd	GCAAGCATTACCAGGCCTATGCCGCCGCACGCGACTTCGC
LP61	CD28 Lck binding motif mutation 3x Pro to Ala rev	CGAAGTCGCGTGCGGCGGCATAGGCCTGGTAATGCTTGCG

2.1.3 Bacteria, cell lines and primary cells

Table 9: Bacteria

Name	Cell type	Source
E. coli TOP10	F^-, mcrA, Δ(mrr-hsdRMSmcrBC), Φ80lacZΔM15, lac224, deoR, recA1, araD139, Δ(ara-leu) 7697, galU, galK, rpsL (Str^R), endA1, nupG	Invitrogen/Life Technologies, Darmstadt, Germany

Table 10: Cell lines

Name	Number	Source
HEK293T/17	CRL-11268	American Type Culture Collection (ATCC), Manassas/VA, USA
HuT78	TIB-161	American Type Culture Collection (ATCC), Manassas/VA, USA
Raji	CCL-86	American Type Culture Collection (ATCC), Manassas/VA, USA
J-Lat_8.4	NIH 9847	National Institutes of Health (NIH), USA

Table 11: Primary Cells

Name	Cell type	Source
buffy coats	human whole blood	German Red Cross Blood Donor Service Baden-Württemberg Hessen, Frankfurt/Main, Germany
Fresh blood of *Macacca mulatta* and *African Green Monkey*	non-human primate whole blood	Department 4, Veterinary Medicine, Paul-Ehrlich-Institut, Langen, Germany

2.2 Methods

2.2.1 Cloning of CAR expression plasmids

CAR-expression plasmid #1138 pBullet (Willemsen *et al.* 2003) was used as a template to build an expression cassette in which single CAR domains can be exchanged individually. Primers were designed, complementary to the CAR domains and adding restriction sites and Myc- or HA-tags. In two initial PCR runs, the CAR binding and spacer domains were amplified and subsequently 5 µl of each of the PCR products were used as a template for the third fusion PCR run (Figure 11). For all PCRs, KOD hot start polymerase kit was used, according to manufacturer's protocol (Table 12).

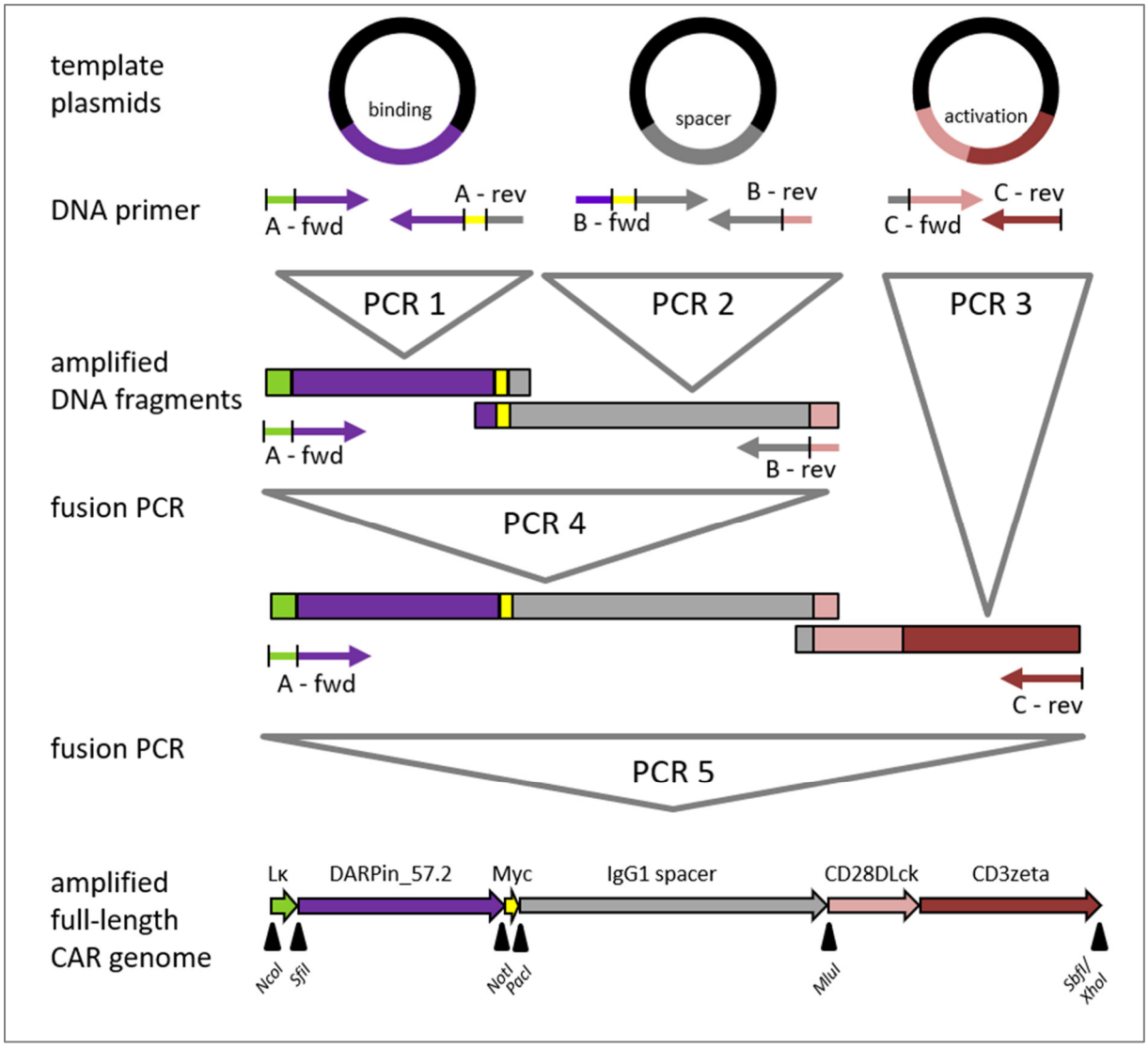

Figure 11: Strategy for cloning of CAR constructs.

CAR fragments were amplified using respective template plasmids and primers encoding restriction sites and tags. Amplified fragments with DNA overlaps served as templates for additional fusion PCR runs, adding primers respectively.

The DNA products were then separated over a 1% agarose gel and the fragment of the required size was purified with NucleoSpin® Gel and PCR Clean-up kit. The amplified DNA fragment was cloned into a TOPO blunt subcloning plasmid and sequenced by Sanger light run tube (GATC Biotech). The entire CAR fragment could then be transferred to the pBullet backbone via restriction sites *BamHI* and *XhoI* using quick T4 DNA ligase.

Table 12: PCR reagents mix

Left: Mix of PCRs 1, 2, 3. Right: Mix of fusion PCR.

reagent	volume
10x buffer	5 µl
$MgSO_4$	3 µl
dNTPs (2 mM each)	5 µl
KOD hot start polymerase	1 µl
H_2O	32 µl
DNA template (10 ng / µl)	1 µl
fwd primer (10 µM)	1.5 µl
rev primer (10 µM)	1.5 µl

reagent	volume
10x buffer	5 µl
$MgSO_4$	3 µl
dNTPs (2 mM each)	5 µl
KOD hot start polymerase	1 µl
H_2O	23 µl
template from PCR1	5 µl
template from PCR2	5 µl
fwd primer (10 µM)	1.5 µl
rev primer (10 µM)	1.5 µl

2.2.2 Generation of CAR transfer vector particles

For production of retroviral vector particles, 2 x 10^7 HEK293T/17 cells in 20 ml DMEM + 10%FCS + 2mM-Q were seeded per T175 cell culture flask. On the following day, these cells were transiently transfected with retroviral vector plasmids using PEI (Longo *et al.* 2013). For each flask, 2.3 ml PEI mix and plasmid mix were produced as follows (Table 13).

Table 13: PEI transfection mix

	reagent	volume
Plasmid mix	pHIT60 (1 µg/µl)	13.70 µl
	pcDNA-GaLVwt (1 µg/µl)	6.09 µl
	transfer-vector-plasmid (1 µg/µl)	15.22 µl
	DMEM (w/o FBS)	2265.00 µl
PEI mix	PEI (18mM)	140.00 µl
	DMEM (w/o FBS)	2160.00 µl

Each mix was vortexed for 20 seconds and incubated at RT for 5 minutes before merging them. After additional 20 seconds of vortexing, PEI-DNA mix was allowed to form during 20 minutes at RT. Medium was then removed from HEK293T cells, except for 7.4 ml. 4.6 ml of PEI-DNA mix were added to the cells, mixed gently with the remaining media. After 6 hours of incubation at 37°C, media was fully replaced with 17 ml DMEM + 10%FCS + 2mM-Q. 48h post transfection, particles were harvested into 50 ml tubes. Following centrifugation at 500x g and 4°C for 10 minutes to remove large cell fragments, supernatant was filtered through sterile

0.45 µl PVDF vacuum filter membranes and stored at -80°C.

2.2.3 Detection of MLV-gag p30 protein by Western Blot

Protein concentration of sterile filtered medium containing retroviral particles (Section 0) was determined by Bradford assay using Bradford reagent (Sigma) as described by manufacturer. 15 µg protein samples were prepared by adding NuPAGE LDS Sample Buffer (4x) and NuPAGE Sample Reducing Agent (10x), each to 1x final concentration. Protein denaturation was performed at 70 °C for 10 min. NuPAGE Bis-Tris gradient gels were washed with ddH_2O before placed into Western Blot chamber with 1x MOPS running buffer (buffer in inner chamber was supplied with 0.25% NuPAGE Antioxidant). Protein samples and 7 µl and Protein marker XXL DeLuxe (GeneOn) were loaded onto the gel and run at 200 V for 55 minutes.
For Western blotting, 500 ml NuPAGE Transfer Buffer (1x) was prepared with 25 ml NuPAGE Transfer Buffer (20x) + 0.5 ml NuPAGE Antioxidant + 50 ml methanol + 24.5 ml ddH_2O. Prior to blotting, PVDF membrane was activated in methanol for 10 s and rinsed with ddH_2O before placed into 1x NuPAGE Transfer Buffer. Protein transfer from the gel to the activated membrane, both were blotted on ice in 1x NuPAGE Transfer Buffer at 35 V for 1h. The membrane was then rinsed in TBST (1x) and blocked in 1x TBST containing 5% powdered milk and 20% horse serum for 3 h at 4°C. Blocking solution was discarded before adding primary antibody, anti-MLV p30 (ATCC) at a 1:10,000 dilution in TBST (1x) + 5% powdered milk + 20% horse serum, overnight at 4°C. The following day, membrane was washed three times with TBST (1x). Secondary anti goat HRP was used at a 1:5,000 dilution in TBST (1x) + 5% powdered milk. After 2 h incubation, membraned was again washed three times with TBST (1x) before developed with Amersham ECL Prime Western Blotting Detection Reagent and High-Performance chemiluminescent films (GE Healthcare) at an exposure time of 3 minutes.

2.2.4 Cell characterisation by flow cytometry

Cells were washed twice with chilled FACS buffer and seeded into 96-v-bottom plates. Antibodies and isotype controls were added as recommended by manufacturer, mixed and incubated at 4°C for 30 min. After two additional wash steps with 200 µl FACS buffer (350x g, 4°C; 10 min) cells were fixed in 50 µl 2% PFA. If analysis was not performed on the same or following day, PFA was replaced by 50 µl FACS buffer and stored at 4°C. Fluorescence of cells was determined using LSRII (BD).

2.2.5 Purification and activation of primary T cells

One day prior to PBMC purification, unlabelled anti-CD3 and anti-CD28 antibodies were diluted in PBS to 3 µg/ml each. For human T cells, anti-CD3 antibody clone OKT-3 was used, for nhps clone SP34.2. 6-well plates were coated with 1 ml antibody dilution per well and incubated overnight at 4°C. The following day, the coated plates were blocked with PBS + 2%BSA at room-temperature for 30 minutes and washed twice with 3 ml PBS. For isolation of PBMCs, serum-free buffy coats were diluted 1:3 with PBS before 30 ml were carefully topped onto 15 ml Histopaque 1077, keeping two liquid phases separated. Ficoll centrifugation was run at 974x g for human and 1028x g for nhp T cells, room-temperature, for 30 min without acceleration and deceleration. The white ring of lymphocytes was transferred to a fresh tube and topped-up to 50 ml with PBS, followed by centrifugation at 500x g for 10 min. To lyse remaining erythrocytes, cells were resuspended in 10 ml pre-warmed ammonium chloride (0,86% NH_4Cl) and incubated at 37°C for 10 minutes. After two additional wash steps in PBS, up to 3 ml cell suspension per well were seeded into pre-coated 6-well plates (5 x 10^6 cells/ml in RPMI + 10%FCS + 2mM-Q + 1%PenStrep + 500 U/ml ProleukinS). If activated T cells were used for heterologous depletion of target cells, $CD4^+$ cells were removed by depletion with magnetic beads (negative depletion with CD4 MicroBeads, human, Miltenyi using AutoMACS cell separator). For autologous CD4 T cell depletion, this step was skipped and cells seeded directly. During incubation for 4 to 6 days, cells were checked daily and medium was topped-up or exchanged as required.

2.2.6 Transduction of primary T cells with gamma-retroviral particles

To transduce activated T cells with retroviral vectors, 24-well plates were coated with PLL (Sigma), 1-3 days prior to transduction. PLL was used at 0.1 mg/ml and 300 µl per well were incubated at 4°C overnight. Activated T cells were centrifuged at 350x g and resuspended to 2 x 10^6 cells per ml in concentrated medium (60% FBS + 40% RPMI + 2mM-Q + 5% PenStrep + 2500 U/ml ProleukinS + 24 µg/ml Protamine sulfate (Sigma)). PLL was removed from coated plates which were then washed with 1 ml PBS. 250 µl cell suspension (0.5 x 10^6 cells) were seeded per well and topped up with 1 ml vector containing supernatant. Spinocculation was performed at 800x g and 32°C for 90 min, followed by incubation at 37°C for additional 90 minutes. Subsequently, 800 µl supernatant were removed carefully and replaced by 500 µl pre-warmed medium (RPMI + 10% FCS + 2mM L-Glut + 1% PenStrep + 500 u/ml Proleukin S). To enhance efficiency, transduction was repeated the following day: After medium was removed and cells topped with 250 µl concentrated medium (see previous day) and 1 ml vector. Following repetition of spinocculation and incubation, all cells of the same type (donor and

vector) were pooled, centrifuged at 450 x g for 20 minutes and diluted to 5 x 10^6 cells/ml in full medium containing 500 U/ml ProleukinS.

2.2.7 Autologous depletion of $CD4^+$ T cells

For autologous depletion of $CD4^+$ T cells, activated and transduced PBMCs were diluted to 5x10^6 cells/ml in medium containing 500 u/ml ProleukinS. 300 µl cell suspension (1.5x10^6 cells) per well were seeded into 48-well plates. Approximately 1.5 million cells per sample were fixed just before transduction. For each additional time point, one well per sample was fixed and stained as described in Section 2.2.4. Samples were stained for expression of CARs using anti-human IgG antibody, which binds to the CAR spacer domain. T cell marker CD3 was used to determine the amount of T cells and within this population, the proportion of CD4 and CD8 positive cells was analysed.

2.2.8 Heterologous depletion of $CD4^+$ cell lines

After isolation of PBMSs from human peripheral blood, $CD4^+$ cells were depleted and CD4 negative cells were activated and transduced with retroviral vectors (Sections 2.2.5, 2.2.6). Expression of CARs was determined in flow cytometry staining the human IgG spacer domain 48h post transduction. If CARs were expressed sufficiently, cells were washed twice with a suitable volume of PBS (350x g, 5 min) and cultured in medium containing 25 U/ml ProleukinS for additional 48h. This step was then repeated and decreased T cell activation was confirmed by reduced expression of early and late activation markers CD69 and CD25 in flow cytometry. Directly prior to co-culture with target or non-target cells, the expression of CARs was again measured in flow cytometry. Within one donor, the proportion of CAR-expressing cells was adjusted to the lowest ratio between all constructs, by adding untransduced T cells of the same donor. To be able to distinguish between T cells and target or non-target cell lines HuT78, J-Lat and Raji, these cell lines were stained with CFSE or violet membrane dye, according to manufacturer's protocol. For co-culture assays, silenced CAR-T cells were added at respective effector:target ratios to 30,000 target or non-target cells at a total volume of 200 µl per well in RPMI containing 10% FBS, 2mM-Q, 1% PenStrep and 25 U/ml ProleukinS. CAR positive cells were defined as effector cells. Control T cells which were transduced with mock control vector, were added at the same number of total T cells as the CAR positive samples. Each sample was prepared in biological duplicates. After incubation at 37°C for 48h, supernatant was frozen in aliquots at -80°C. Cells were washed twice with 250 µl FACS buffer and fixed in 100 µl 2% PFA. Flow cytometric analysis was performed using MACSQuant® Analyzer 10. Therefore, the total number of remaining target and non-target cells per well was counted. For each

experiment, T cells of two different donors were used in parallel and each experiment was repeated three times with new donors, resulting in a total of n=6 donors, unless stated otherwise. In order to determine cytotoxicity [%], the average number of remaining target cells between biological duplicates was normalised to the average number of target cells remaining in the samples using the same donor and the same effector:target ratio.

$$cytotoxicity\ [\%] = \frac{average\ remaining\ target\ cells\ (sample)}{average\ remaining\ target\ cells\ (mock\ control)}$$

Specific T cell activation was determined by secretion of interferon-γ in the co-culture supernatant using interferon-γ ELISA (Mabtech).

2.2.9 Titration of compounds inducing HIV expression of J-Lat cells

In this project, J-Lat cells were used as a model for HIV latency. They are based on a clone of Jurkat cells which was stably transduced with a full-length HIV-1 genome in which the *env* gene was mutated by a frameshift and *nef* is replaced by GFP. Upon activation, the HIV genome is transcribed and translation can be measured through expression of GFP. In order to find the optimal cell stimulus for cell viability and activation, 50,000 J-Lat cells were seeded per well in 200 µl RPMI containing 10%FBS and 2 mM-Q. Compounds were added to final concentrations as followed:

Prostatin:	50 µM	→	10 µM	→	2 µM	→ 0.4 µM
SAHA:	25 µM	→	5 µM	→	1 µM	→ 0.2 µM
Prostatin/SAHA:	50/25 µM	→	10/5 µM	→	2/1 µM	→ 0.4/0.2 µM
Interferon-γ:	100 µM	→	20 µM	→	4 µM	→ 0.8 µM

For all samples, two plates were seeded in duplicates for each sample. Cells were incubated at 37°C and 5% CO_2. One plate was analysed after 24h and one plate after 48h. As a positive control for dead cell dye, 100,000 J-Lat cells were incubated at 65°C for 15 minutes and stained like all other samples. Therefore, cells were washed twice in 300 µl PBS and centrifuged at 350x g and 4°C. Cells were then resuspended in 100 µl PBS containing 0.1% Fixable Viability Dye eFluor™ 780 (eBioscience). After 30 minutes of incubation at 4°C in the dark, cells were again washed twice with 300 µl FACS buffer, followed by overnight fixation at 4°C in 100 µl PFA (4%). Flow cytometry analysis was performed in MACSQuant Analyzer 10 (Miltenyi) using signals of channel B1 (gfp) for activated cells and R2 (APC eFluor) for dead cells.

2.2.10 Heterologous depletion of HIV⁺ target cells

Depletion of heterologous HIV⁺ target cells was performed as described in 2.2.8, as duplicate design. J-Lat cells were labelled with CellTrace™ Violet Cell Proliferation Dye (Invitrogen/Fisher) and T cells of two donors were used per experiment. After 48h, one co-culture replicate was analysed in flow cytometry and IFN-γ ELISA. In the second replicate, 100 µl of supernatant were removed and 100 µl medium containing 10 µM Prostatin and 5 µM SAHA. Given that 100 µl medium still remained in the well, compound dilution factor was 2 and therefore lead to final concentrations of 5 µM Prostatin and 2.5 µM SAHA. Cells were then incubated with the compounds for additional 36h before analysis in flow cytometry (Figure 12 A).
In an additional experiment (Figure 12B), 30,000 J-Lat cells per well were first activated with 5 µM Prostatin and 2.5 µM SAHA for 36 h. After gfp expression was confirmed with using a fluorescence microscope, activated J-lat cells were added to the CAR T cells at different ratios and incubated for 48 hours as described (Section 2.2.8). Fixed cells were analysed with MACSQuant Analyzer 10 (Miltenyi).

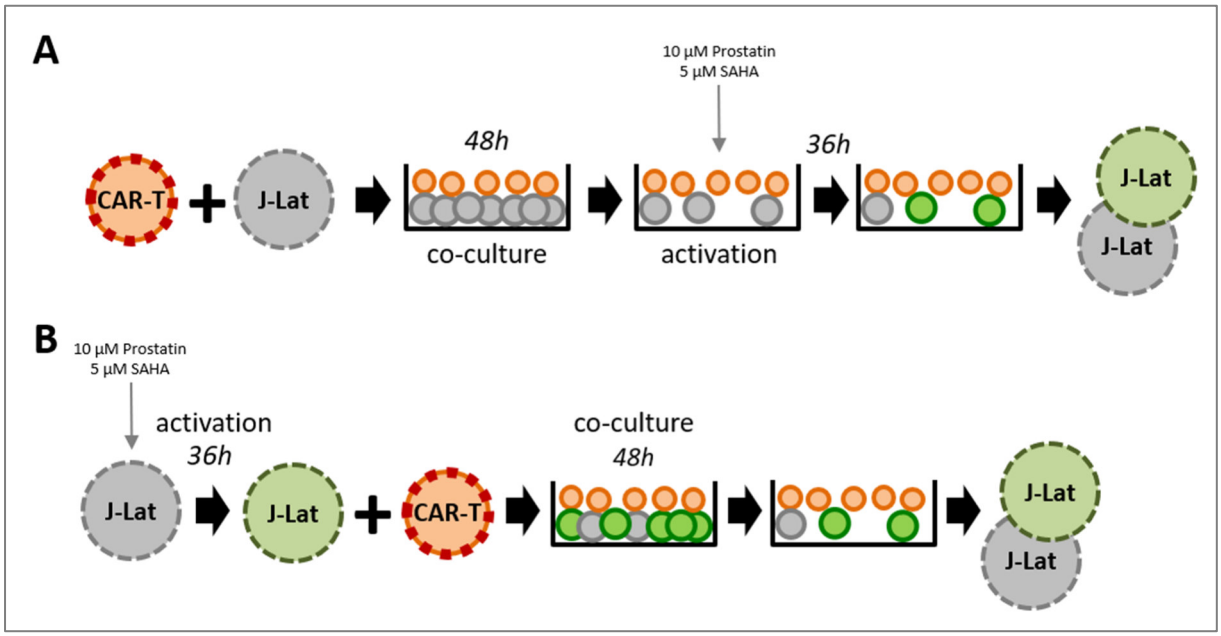

Figure 12: Experimental layout of depletion of HIV⁺ target cells.

Upon activation with Prostatin and SAHA, J-Lat cells express HIV-eGFP (green). After each experiment, the number of GFP⁺ and GFP⁻ J-Lat cells was determined by flow cytometry. **(A)** J-Lat cells were co-cultured with CAR-T cells for 48h, before activation for additional 36h. **(B)** J-Lat cells were activated for 36h before co-culture with CAR-T cells.

3 Results

3.1 Generation of CAR-expressing T cells

Human PBMCs were isolated from whole blood. T cells were specifically activated with anti-CD3 and anti-CD28 antibodies before transduction with MLV gamma-retroviral particles. These vector particles were generated by transduction of HEK293T cells with a three-plasmid system consisting of MoMLV expression plasmid pBullet, which encodes the CAR genome, pHIT60 which encodes MLV gag-pol polyprotein and pCOLT, encoding GaLV env protein.

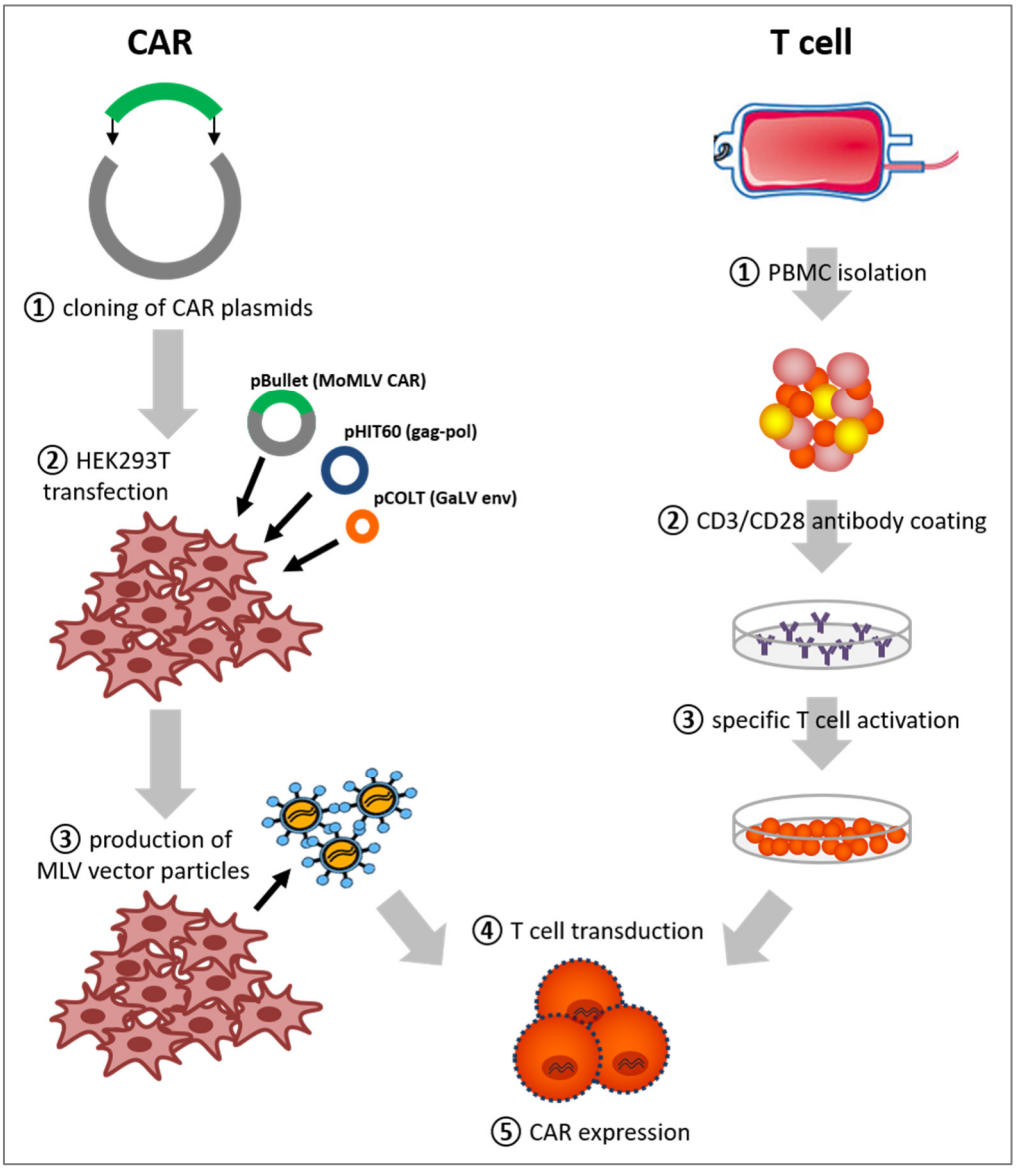

Figure 13: Production of CAR-T cells.

Work stream **A**: After cloning of MLV expression plasmids encoding the CAR construct of choice **(1A)**, these are co-transfected with gag-pol and env plasmids into HEK293T producer cells **(2A)**. Retroviral particles are harvested 48h post transfection **(3A)**. Work stream **B**: PBMCs are isolated from human blood **(1B)** and specifically activated with anti-CD3/anti-CD28 antibodies **(2B, 3B)**. Activated T cells are transduced with retroviral particles **(4)** and expression of CARs is determined 48h post transduction **(5)**.

3.1.1 Cloning of CAR expression plasmids

In order to generate CARs with different binding domains, a framework was inserted into the original expression plasmid #1138 pBullet. PCR primers were designed to add restriction sites for single-cutting enzymes between all CAR domains. Myc or HA tags were added aiming to detect CARs on the cell surface with monoclonal antibodies (see Section 2.2.1 for strategy). To redirect T cells towards $CD4^+$ T cells, the extracellular scFv domain of a CAR was replaced by a DARPin targeting human CD4.

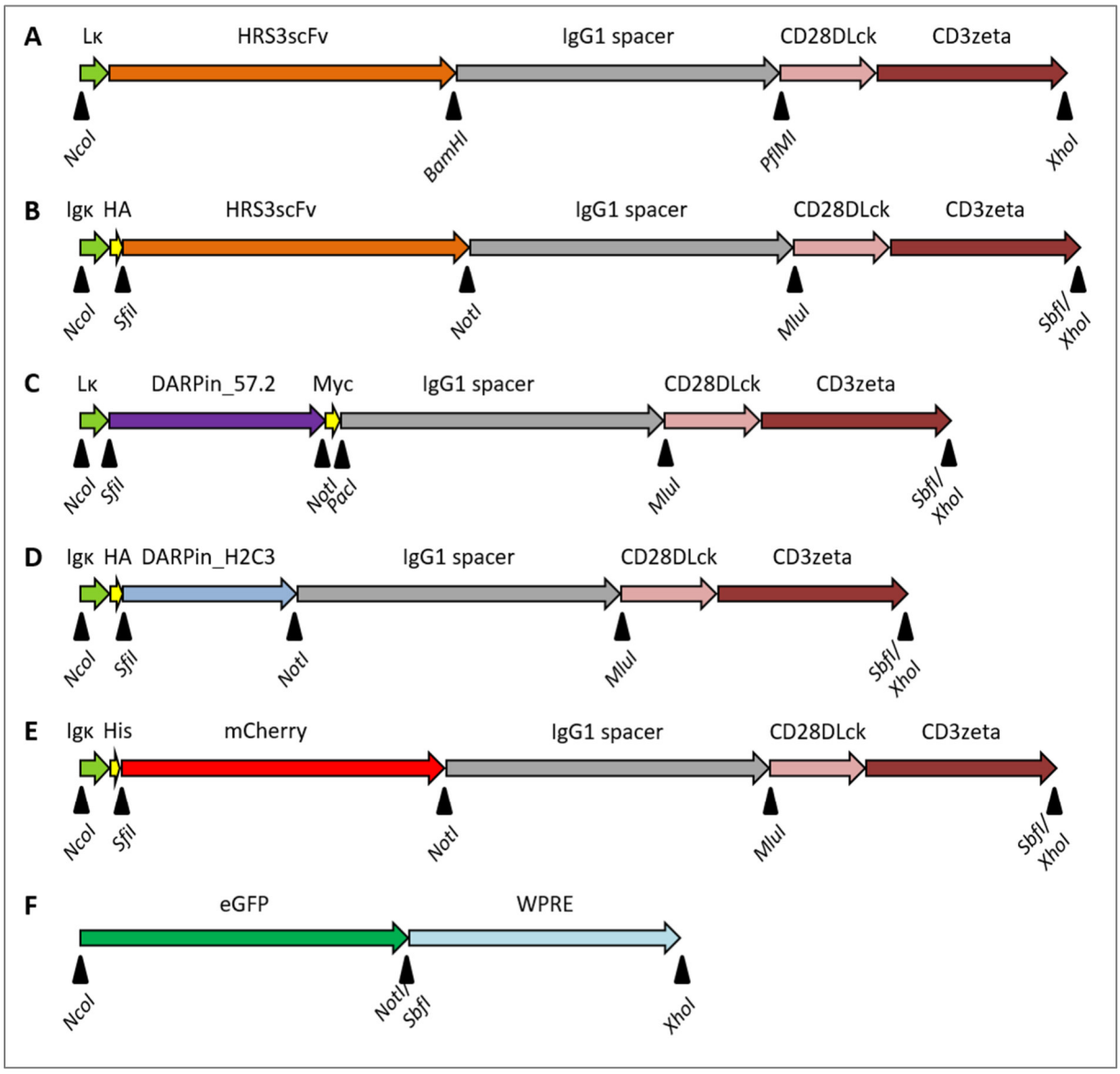

Figure 14: Generation of various CAR expression plasmids.

(A) Original CAR genome #1138 pBullet encoding HRS3scFv binding domain targeting human CD30. **(B)** Anti-CD30 CAR with additional HA-tag and restriction sites. **(C)** Anti-CD4-DARPin CAR. **(D)** CAR containing PBMC-unspecific binding domain DARPin_H2C3. **(E)** CAR binding domain replaced by mCherry fluorescent protein. **(F)** CAR substituted by enhanced GFP.

Figure 14 gives an overview of the various CAR constructs which were generated for this work and which all follow the same framework. The leader sequences, Lκ or Igκ, were followed by the extracellular CAR binding domains scFv or DARPin or the fluorescent protein mCherry. The extracellular/transmembrane domain consisted of the identical IgG1 spacer throughout all constructs. All constructs were terminated by an intracellular CD28 co-stimulation and CD3zeta activation domain, which was also not changed between any of the different CARs. Figure 14F shows the eGFP genome enhanced by a WPRE site, which was cloned into the MLV expression plasmid instead of a CAR to determine transduction efficiency of the produced MLV particles for different donors.

Because KOD hot start polymerase was used for PCR, amplified DNA products contained blunt ends. Linear DNA was therefore subcloned into a TOPO zero blunt plasmid which contained binding sites for standard primers T7 and M13 which were used to determine the full sequence of the amplified insert. After confirmation of the correct sequence, the TOPO-CAR gene plasmids were digested with restriction enzymes *NcoI* and *XhoI* to receive a linear CAR fragments with sticky ends which could then be separated over agarose gels (Figure 15A). Purified DNA was then ligated into the backbone of MLV expression plasmid. Insertion of the correct CAR genome was determined by analytical enzymatic DNA digestion. Presence of a DARPin binding domain was confirmed by restriction enzymes *XhoI* and *BlpI*. A restriction site for *BlpI* was only present in HRS3scFv, resulting in two DNA fragments. On the contrary, DARPin plasmids resulted in one large linear fragment around 7.23 kb (Figure 15B).

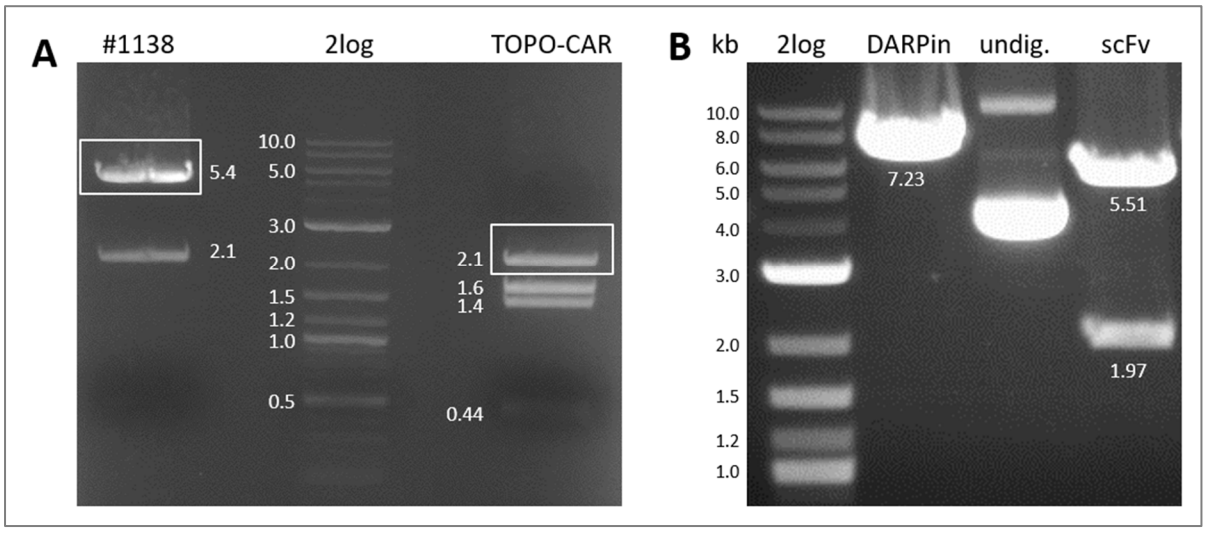

Figure 15: DNA fragments of CAR expression plasmids after enzymatic digestion.

(A) Digestion of original CAR expression plasmid #1138 pBullet-Lk-HRS3scFv-CD28DLck-CD3zeta and TOPO-IgK-HA-HRS3scFv-DFc-huCD28DLck-CD3zeta, digested with restriction enzymes *NcoI* and *XhoI*. Fragments of 5.4 kb (backbone) and 2.1 kb (insert) before ligation of new pBullet-IgK-HA-HRS3scFv-DFc-huCD28DLck-CD3zeta. **(B)** Analytical digestion of CAR expression plasmids containing anti-CD4-DARPin_57.2 (left) or antiCD30scFv (right) binding domains, with enzymes *XhoI* and *BlpI*. Undigested DARPin plasmid is shown in the centre lane.

3.1.2 Generation of CAR transfer vector particles

Gamma-retroviral vectors pseudo-typed with the gibbon ape leukaemia virus (GaLV) envelope protein were used for the genetic modification of T cells. To generate transfer vector particles, HEK293T cells were co-transfected with CAR encoding MLV expression plasmid pBullet, pHIT60 encoding MLV gag-pol and pCOLT containing env gene of GaLV. The protocol for production of retroviral particles was established using MLV transfer vector plasmids encoding fluorescent eGFP or mCherry CAR. In these cases, successful transfection of HEK293T producer cells could be confirmed directly by fluorescence microscopy.

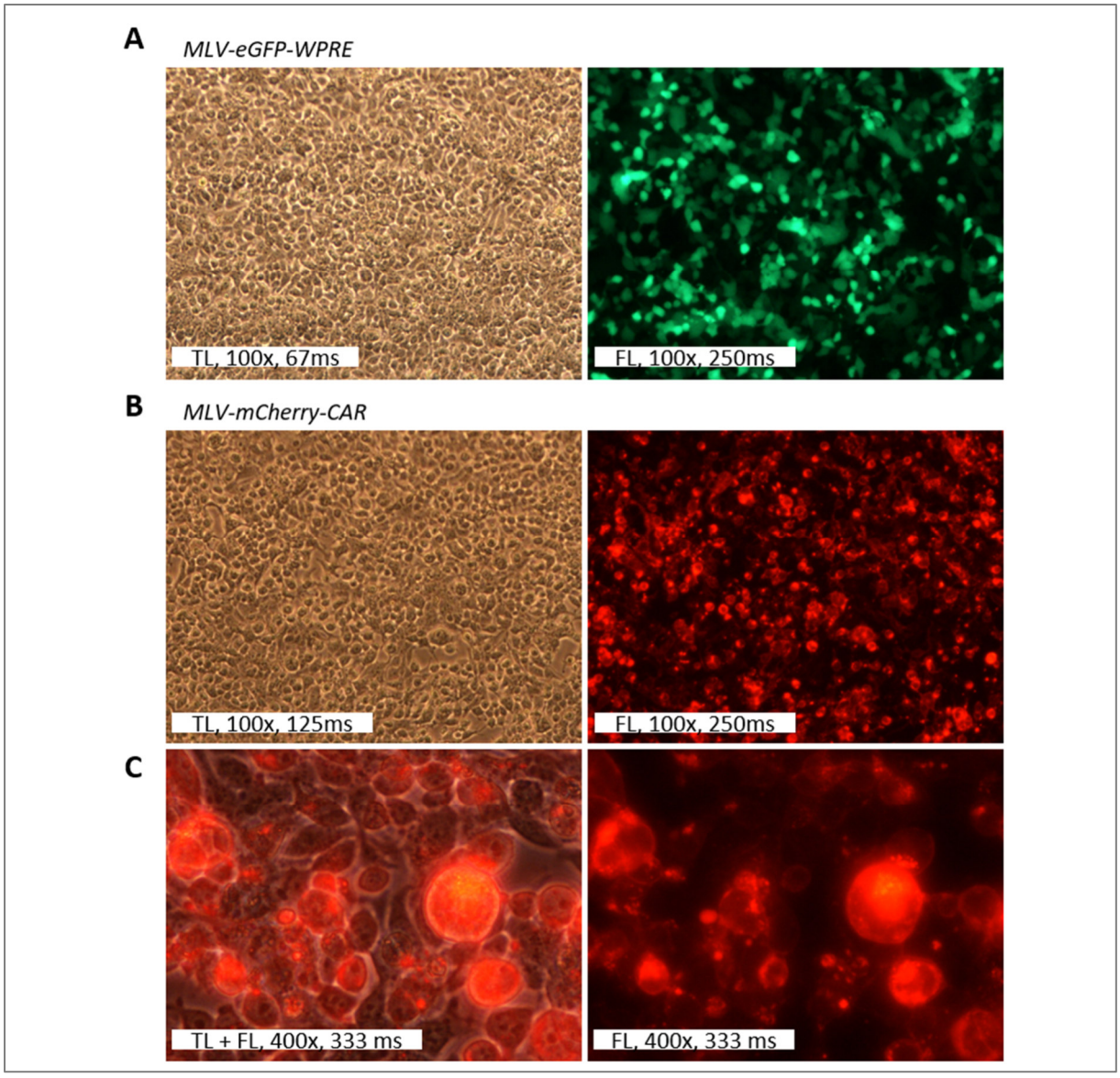

Figure 16: HEK293T cells producing gamma-retroviral particles.

HEK293T cells 48h post transfection with MLV-eGFP **(A)** or MLV-mCherry-CAR **(B + C)**.
TL = transmitted light, FL = fluorescent light

Figure 16 shows the successful transfection of HEK293T producer cells with a 3-plasmid system, consisting of the MoMLV gene transfer vector plasmid based on #1138 pBullet (Hombach *et al.* 1998), pHIT60 and pcDNA-GaLVwt. Expression of fluorescent proteins eGFP (A) and mCherry (B + C) was determined by fluorescence microscopy. After establishment of the transfection protocol (Section 0), multiple MLV-based retroviral particles were produced. Vector particles, in which the gene-transfer plasmid was replaced by non-coding pcDNA served as mock control. 48h post transfection, supernatant was collected from HEK293T cells, filtered and frozen at -80 C.

To confirm the presence of retroviral particles, supernatant was applied to Western blotting. Vectors encoding the respective CARs or mock control were detected by expression of MLV-gag-p30 (Figure 17).

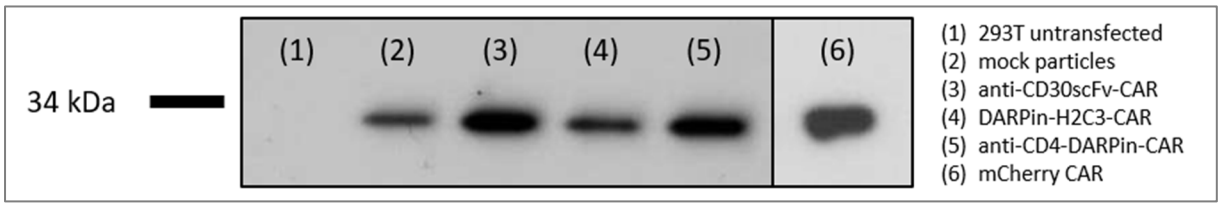

Figure 17: Expression of MLV-gag protein p30 by γ-retroviral vectors.

Expression of MLV-gag protein p30 by γ-retroviral vectors in supernatant of transfected HEK293T/17 cells: Particles used for T cell co-culture assays: empty mock control (lane 2) or CAR delivering vector particles (lanes 3-6), supernatant of untransfected cells served as negative control (lane 1).

MLV-gag-p30 could be detected for all produced vector batches.

3.1.3 Isolation and activation of primary T cells

Human PBMCs were isolated from whole blood using ficoll-gradient centrifugation. Cells were washed with PBS and seeded on plates, pre-coated with anti-human CD3 and CD28 antibodies. Culture medium was supplemented with 500 u/ml ProleukinS, a derivate of human IL-2, to stimulate T cell proliferation. After 3-6 days, depending on the appearance of clump formation, purity of the population was assessed by detection of T cell marker CD3 and activation by CD25. Expression of both markers was assessed by flow cytometry.

Figure 18 demonstrates expression of T cell marker CD3 within the population of activated PBMCs after 3 days. Donor 3 achieved a T cell purity of 84.1% and donor 2 of 88.2%. Donors 1 and 4 showed an even higher T cell purity of 95.2% and 96.2% respectively.

Specific activation of PBMCs with anti-human CD3 and CD28 antibodies led to a very high purity of the T cell population.

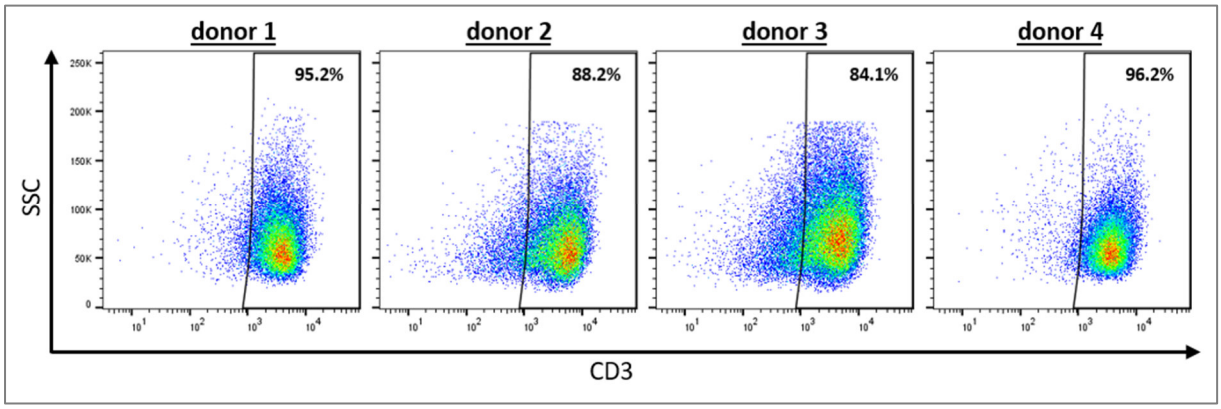

Figure 18: T cell purity after 3 days of activation.

Proportion of CD3 expressing cells within PBMCs activated on tissue culture plates coated with anti-human CD3 and CD28 antibodies for 3 days. 4 representative donors shown in flow cytometry.

Figure 19A (left) show that only minor clumps are formed by cells which have not been activated. In addition to the visual examination of the T cell activation status, expression of CD25 was also determined in flow cytometry, since CD25 is a marker which is upregulated during consistent T cell activation. Figure 19B shows expression of CD25 for two representative donors. Both donors showed high upregulation of CD25 after 3 days of activation with CD3/CD28 antibodies (black line), compared to expression before activation (grey shaded).

After 3 days of activation with anti-human CD3 and CD28 antibodies, human PBMCs differentiated to a nearly pure T cell population in a significantly activated state.

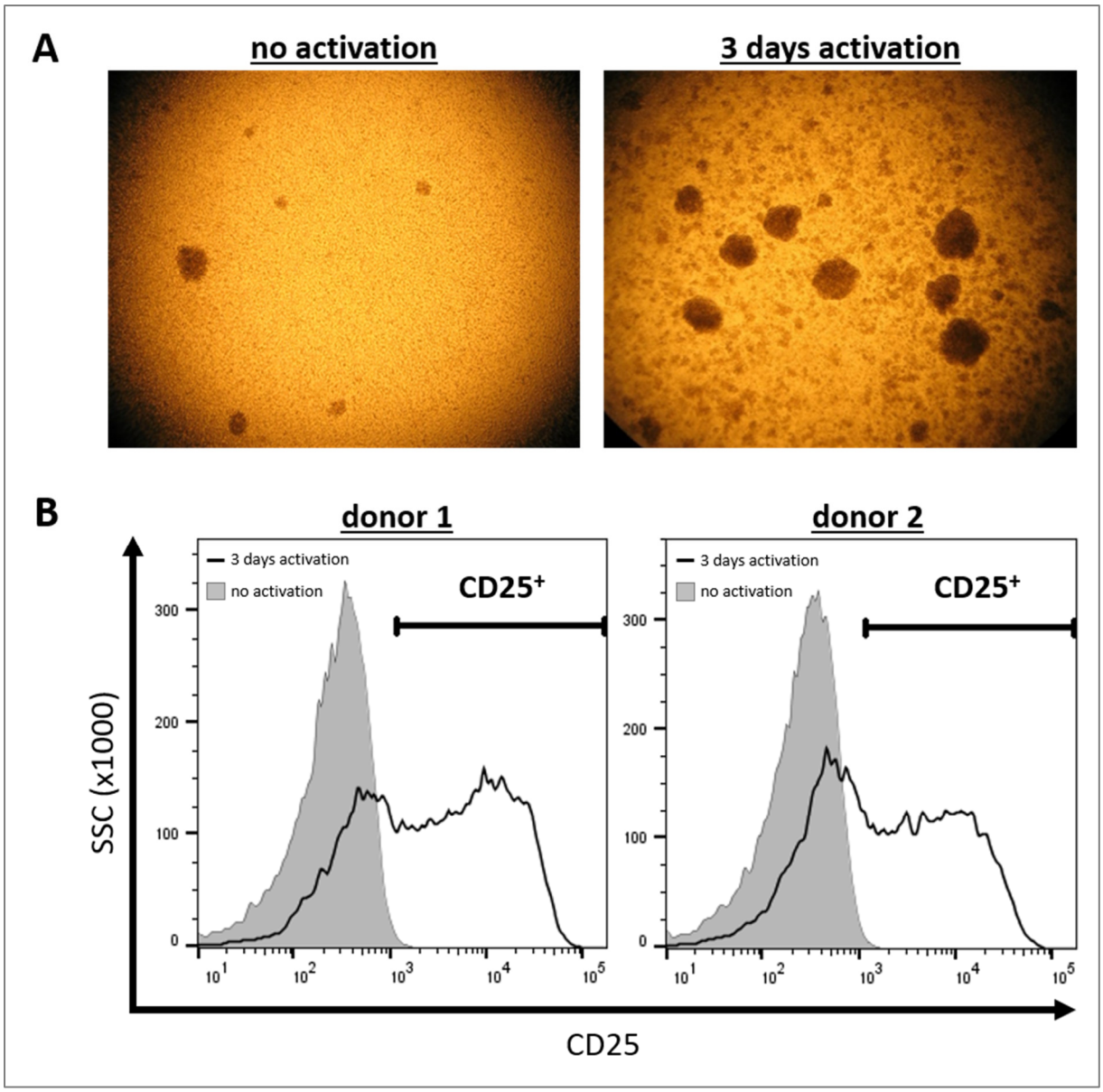

Figure 19: Activation of human T cells with anti-CD3 and anti-CD28 antibodies.

(A) Human PBMCs before (left) and after 3 days (right) of activation on tissue culture plates, coated with anti-human CD3 and CD28 antibodies. Transmitted light microscopy 40x magnification. **(B)** Upregulation of activation marker CD25 before and after 3 days of activation in flow cytometry for two representative donors.

3.1.4 Transduction of human T cells with retroviral particles

After 4-6 days of specific activation, T cells were transduced with gamma-retroviral particles as produced in Section 0. Cells were therefore transferred to PLL-coated 24-well plates, topped up with supernatant containing MLV particles. After 90 minutes of spinocculation, cells were incubated at 37 °C, overnight. The following day, supernatant was replaced by fresh su-per-natant containing retroviral particles of the same batch and spin-occulated as before. After additional 24h incubation, cells were washed in PBS and cells cultured with fresh medium containing 500 u/ml ProleukinS. To confirm the successful transduction of T cells, MLV parti-cles containing fluorescent marker genes served as references as previously described for transfection of HEK293T (Section 3.1.2). Expression of the marker transgenes eGFP or mCherry CAR were detected by fluorescent microscopy.

Figure 20 shows activated and transduced human T cells 48h post transduction with MLV particles encoding eGFP (top) or mCherry CAR (bottom).

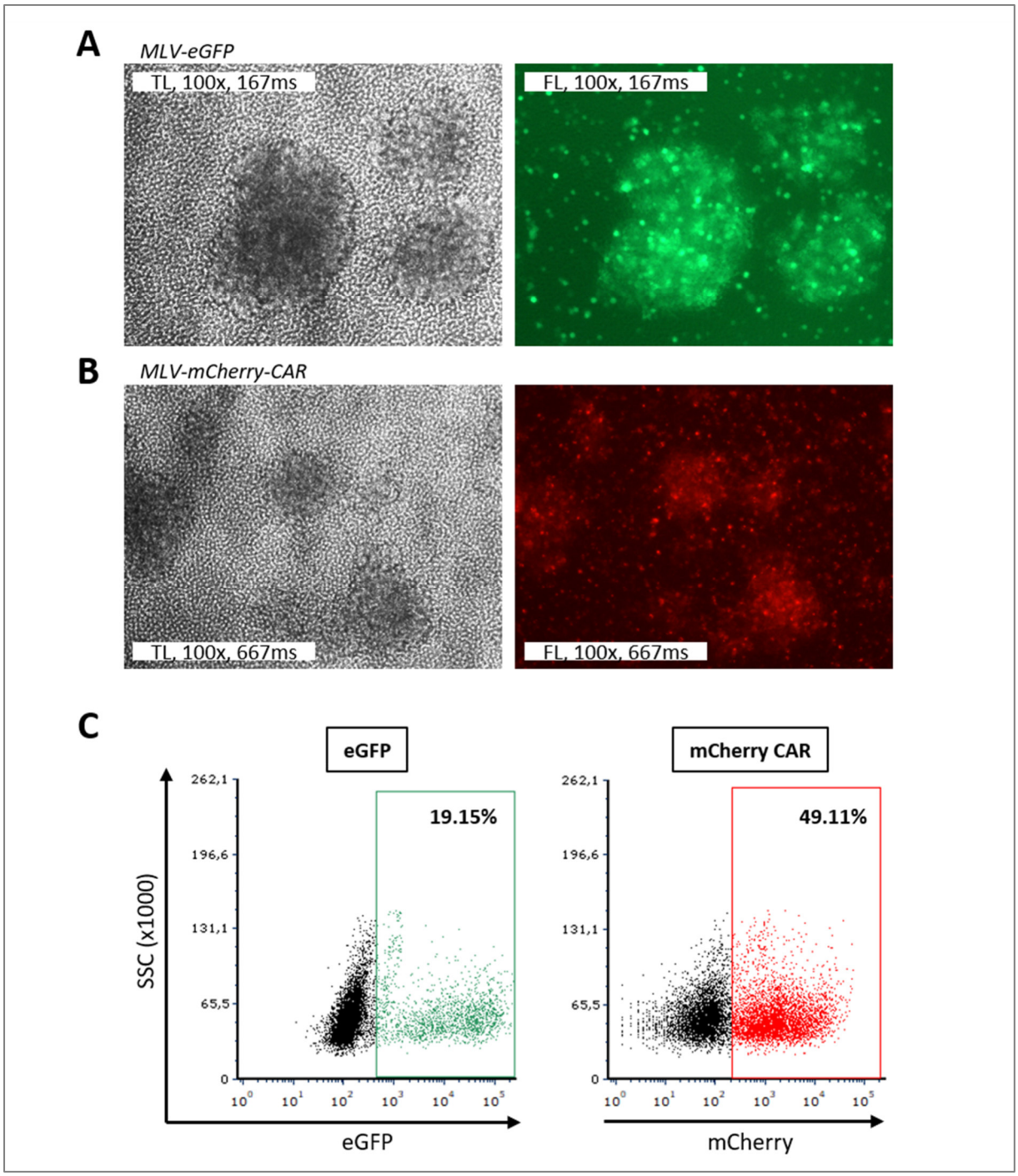

Figure 20: Expression of eGFP or mCherry-CAR by MLV-transduced T cells.

Human T cells expressing eGFP **(A)** or mCherry-CAR **(B)**. Identical image Sections shown using transmitting light (TL, greyscale, left) and fluorescent light (FL, right). **(C)** Detection of GFP or mCherry expressing T cells in flow cytometry, 72h post transduction, compared to unstained mock transduced T cells.

Human T cells were successfully transduced with MLV vector particles leading to expression of the transgene. The established protocol was then used to generate further human T cells expressing various CARs as described in Figure 14.

Since other CARs, used during this work, did not contain any auto-fluorescent proteins (Figure 14), T cells had to be stained with a PE-labelled antibody which binds to the IgG1 spacer domain of the CAR, to determine receptor expression. T cells were therefore stained with Goat F(ab')2 Anti-Human IgG PE antibody or the respective isotype control (Section 2.2.4), 48h post transduction. The number of CAR^+ cells was then determined in comparison to the isotype control-stained sample. MLV particles, in which the transfer vector plasmid had been replaced by pcDNA(-), served again as CAR-negative mock control.

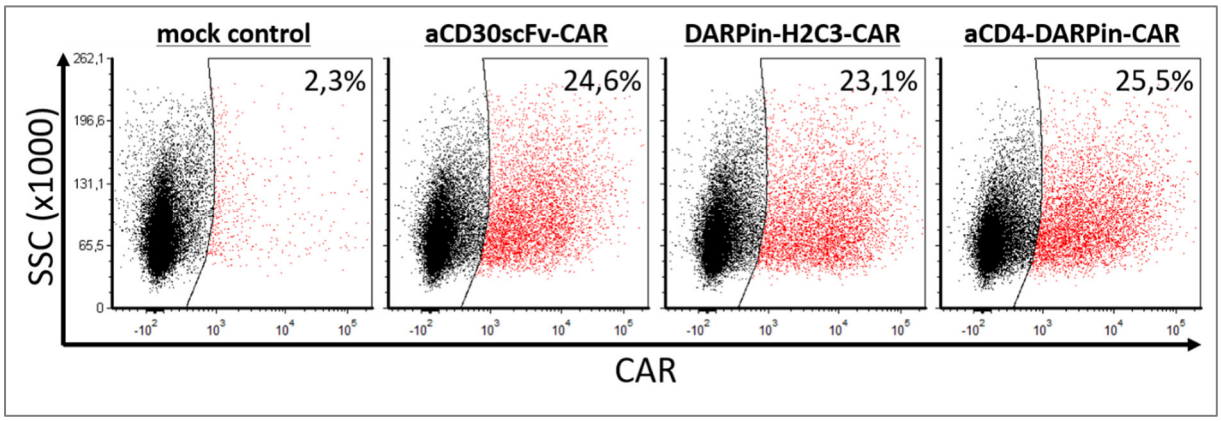

Figure 21: CAR expression on the surface of human T cells.

Expression of CAR containing binding domains CD30scFv, DARPinH2C3 or aCD4DARPin_57.2. Mock control (left) was transduced with particles containing pcDNA only.

Figure 21 shows the CAR staining of one representative donor. Across all experiments and donors, T cell transduction resulted in 15% to 25% CAR^+ cells. The expression of the different CAR constructs within one donor differed by no more than +/- 2.5%.

Human T cells were genetically modified to express DARPin CARs with similar efficiency as scFv CARs using MLV-based particles.

3.2 Evaluation of function and potency of anti-CD4-DARPin CAR-T cells

After DARPin CAR-T cells could be successfully generated, they were tested for target-specificity, potency and efficacy. Binding of a CAR to its particular target antigen leads to a signal transmission to the intracellular receptor domain which then leads to activation of the T cell (Section 1.2.1). This activation is accompanied by the secretion of cytokines, mostly interferon-γ, which is essential for the killing mechanism as well as recruitment and activation of surrounding CAR-T cells. At the same time, this specific CAR-T cell activation needed to be exclusively triggered by the target antigen but not in its absence. In order to determine efficacy, specificity and thereby also safety of the generated anti-CD4-DARPin CAR-T cells, they were co-cultured with CD4 positive and negative target cells.

Because CD4 is a target antigen which is also expressed by the transduced T cells themselves, activation of CD4 specific CAR T cells would have been distorted. Therefore, $CD4^+$ cells were depleted from PBMCs directly after isolation from human blood by magnetic beads. To confirm that the activated cell population did not contain any $CD4^+$ T cells, they were stained with a PE labelled anti-human CD4 antibody and analysed in flow cytometry. Figure 22 shows the CD4 expression of the negative and positive fractions after separation using CD4 specific magnetic beads of one representative donor. Within the CD4 negative fraction (left) only a small $CD4^+$ cell population remained (8.5%). According to the position in the side scatter (SSC), this population is very likely to be monocytes. This hypothesis was also substantiated by the lower MFI (mean fluorescence intensity) of the PE signal compared to the higher signal of the CD4 T cell population highlighted in the plot on the right, showing the CD4 positive fraction. Monocytes are known to express lower amounts of CD4 compared to T cells which then results in a lower MFI. Because after magnetic separation, the CD4 negative fraction was activated with T cell specific anti-CD3 and anti-CD28 antibodies (Section 2.2.5), it was shown before that after 3 days of activation, T cells were the only remaining cell population (Figure 18). Residual $CD4^+$ monocytes were eliminated due to T cell activation.

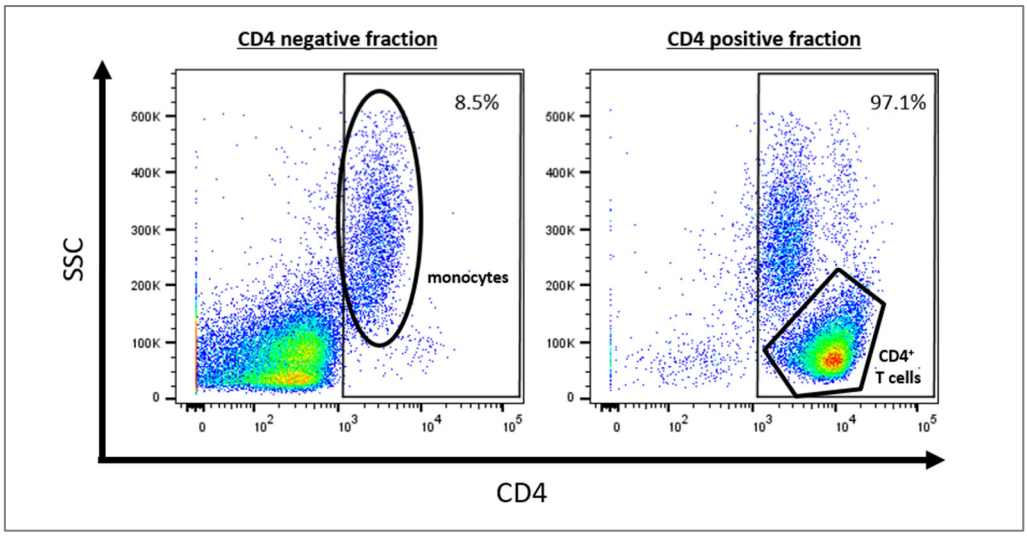

Figure 22: Magnetic depletion of CD4⁺ T cells.

After magnetic separation of CD4⁺ T cells from PBMCs, both fractions were stained for CD4 expression. The CD4 negative fraction (left) and CD4 positive fraction (right) show separation of lymphocyte populations.

The CD4 T cell population could be depleted from human PBMCs by magnetic separation.

3.2.1 Specific activation of anti-CD4-DARPin CAR-T cells and dose-dependent depletion of CD4⁺ target cells

The specificity of DARPin_57.2 for human CD4 was shown previously (Schweizer *et al.* 2008). In order to confirm this specificity, when it is used as a CAR binding domain, DARPin_57.2 was replaced within the CAR construct, by another DARPin, which does not bind CD4⁺ cells. DARPin-H2C3 was shown not to bind human PBMCs, which do contain CD4⁺ cells, while DARPin H2A4 did. (Unpublished DARPins H2C3 and H2A4 kindly provided by C. Buchholz, Pr1/PEI).

Cells of the cutaneous T lymphocyte line HuT78 express high levels of CD4 and CD30 whereas cells of the Raji B cell lymphoma line were negative for both antigens (Supplementary Figure 2). To record specific activation through the engineered CAR upon antigen binding, CAR-T cells were incubated with either HuT78 target cells (Figure 23 A+C) or with Raji cells (Figure 23 B+D). Levels of IFN-γ in the supernatant were detected by ELISA after 48h. Background activation of CAR-T only, 30.000 CAR⁺ T cells without target cells, was assessed within the assay. Secretion of IFN-γ by mock transduced T cells was detected at an average level of 0.075 ng/ml, ranging from 0.242 to 0.003 ng/ml. For all other CAR-T cell, the background activation was found to be at similar levels. The highest average background of 0.465 pg/ml was

produced by aCD30scFc-CAR expressing cells, slightly above aCD4-DARPin CAR-T cells producing an average of 0.30 ng/ml. Further used unspecific DARPin-CAR-T negative control H2C3 was with an average of 0.030 ng/ml even below target cell HuT78 only, which ranged from 0.16 to 0.019 ng/ml, resulting in an average of 0.07 ng IFN-γ per ml supernatant. In presence of HuT78 cells, the extent of CAR-T cell activation, as determined by IFN-γ release, increased with the effector:target cell ratio (Figure 23 A). Both CAR-T cells, anti-CD30scFv and anti-CD4-DARPin produced high amounts of IFN-γ during 48h co-culture with antigen-positive HuT78 cells. Mean levels of IFN-γ secretion by anti-CD30scFv CAR-T cells increased from 0.121 ng/ml at an effector:target ratio of 1:1024 up to 50.28 ng/ml at 1:2 effector:target. Just like anti-CD30scFv, also anti-CD4-DARPin CAR-T cells did not show an increase of IFN-γ release at low E:T ratios, 0.208 ng/ml at 1:1024 E:T. However, with increasing effector rates, anti-CD4-DARPin CAR-T cells achieved a maximum mean IFN-γ level of 43.74 ng/ml at 1:4 E:T. Both antigen-specific CARs started to clearly exceed IFN-γ levels of the negative controls, mock and DARPin-H2C3, at E:T ratio 1:32. To determine T cell activation caused in the presence of antigen-negative cells, CAR-T cells were also co-cultured with Raji non-target cell line (Figure 23 B). Here, both CAR-T cells produced IFN-γ on the same range as the mock control with a mean of 2.132 ng/ml. Anti-CD30-CAR secreted 3.946 ng/ml mean and anti-CD4-DARPin CAR-T cells a mean of 1.765 ng/ml, when incubated with Raji non-target cells.

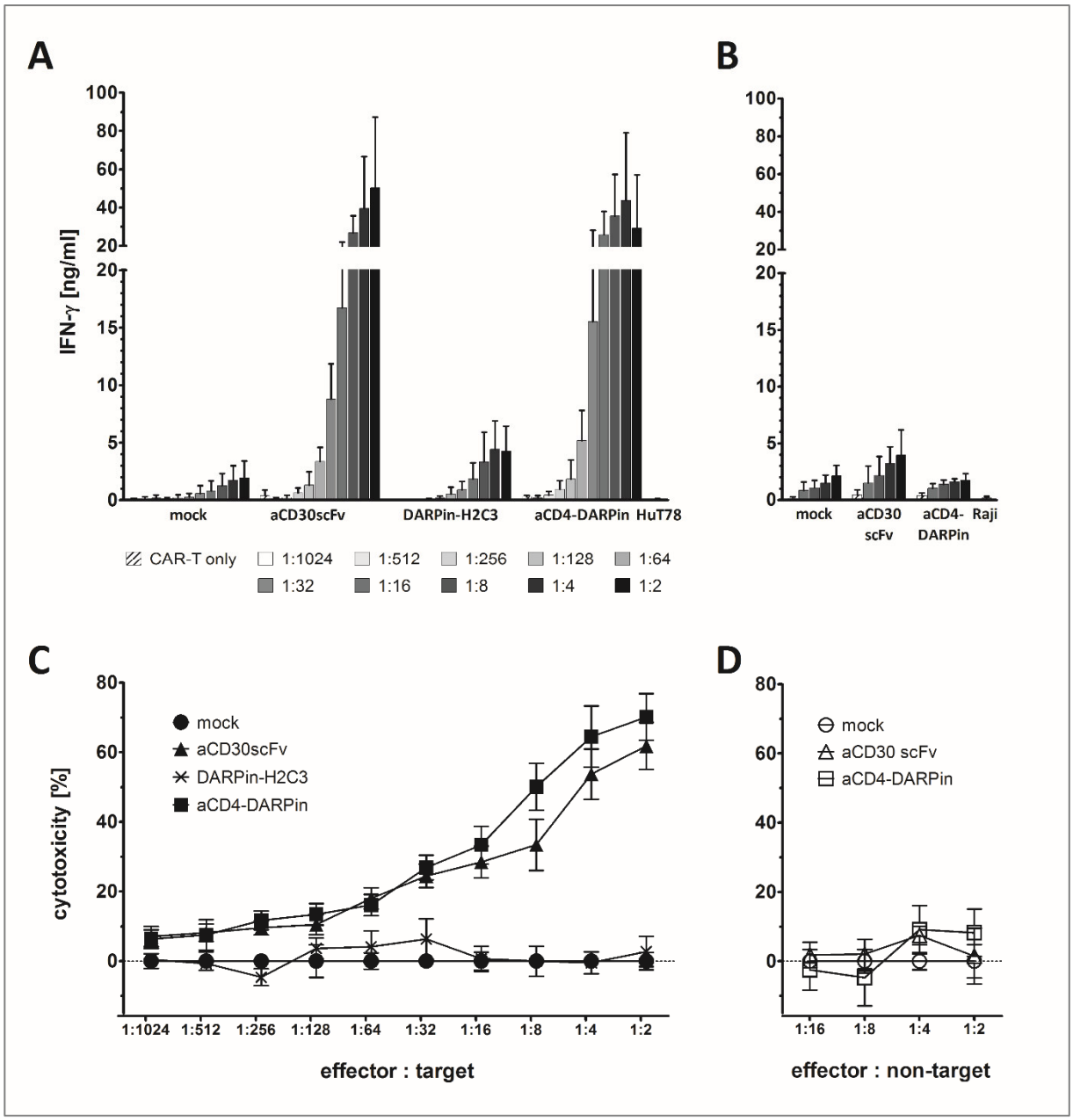

Figure 23: Dose-dependent activation of DARPin-CAR-T cells and depletion of heterologous target cells.

HuT78 target cells **(A, C)** and Raji non-target cells **(B, D)** were incubated with CAR-T cells expressing the anti-CD30scFv-, DARPin-H2C3- or anti-CD4-DARPin-CAR for 48h. **(A, B)** CAR-T cell activation was determined by secreted IFN-γ in supernatant using ELISA. Error bars represent mean standard deviation (SD). **(C, D)** Number of remaining target/non-target cells was determined by flow cytometry and the cytotoxicity [%] calculated in comparison to the respective negative control. Error bars represent standard error of the mean (SEM). Error bars represent SEM. All assays were performed with blood from n = 6 donors in biological duplicates.

In addition to specific T cell activation, cytotoxicity of the generated CAR-T cells was determined in the same co-culture after 48h. Therefore, the number of remaining fluorescently labelled target or non-target cells was measured in flow cytometry. This number was then

normalised towards the average remaining target/non-target cells of the respective mock control of the same effector:target ratio (Figure 23 C+D). Unspecific control DARPin-H2C3 CAR-T cells did not significantly differ from the mock control, -4.6% at 1:256 and +6.3% at 1:32 E:T was the variability for this sample. Already at the lowest effector:target ratio of 1:1024, both antigen-specific CAR-Ts, anti-CD30scFv and anti-CD4-DARPin, had a low mean cytotoxicity of 7.1% and 6.3% respectively. Cytotoxicity was increased alongside with ascending effector:target ratios. At 1:2 E:T, anti-CD30scFv CAR-T cells specifically depleted 61.1% of HuT78 cells and anti-CD4-DARPin CAR-T cells depleted 70.2% of target cells, compared to the mock control. When co-cultured with Raji non-target cells (Figure 23D), values for cytotoxicity were between 1.8% (1:16) and 7.4% (1:4) for anti-CD30scFv and -4.7% (1:8) and 9.1% (1:4) for anti-CD4 DARPin CAR-T cells. A correlation between E:T ratio and cytotoxicity was not observed.

T cells expressing the anti-CD4-DARPin-CAR mediated cytotoxicity towards HuT78 cells in a dose-dependent fashion. As expected, HuT78 cells were also lysed by anti-CD30scFv CAR-T cells (Figure 23 C). In contrast, T cells expressing the DARPin-H2C3 CAR of irrelevant specificity or mock modified T cells did not lyse HuT78 cells. Moreover, the CAR T cells expressing the anti-CD4-DARPin or anti-CD30scFv CAR, respectively, had no effect on Raji cells (Figure 23 D).

It was concluded that anti-CD4-DARPin CAR mediated specific T cell activation towards $CD4^+$ target cells.

3.2.2 Efficient depletion of a rare target cell population by anti-CD4-DARPin CAR-T cells

Reflecting the situation in an HIV-infected patient, the number of cells expressing the CD4 target antigen is expected to be very low. Decline of $CD4^+$ T cells can be caused by viremia or towards the end of depletion by CAR-T cells. This can be problematic since CAR-T cells decrease activation and proliferation in the absence of antigen. To assess the potential of anti-CD4-DARPin CAR-T cells, another co-culture assay was performed using a mixed population of target and non-target cells. Therefore, HuT78 target and Raji non-target cells were mixed in a defined ratio with an increasing amount of HuT78 alongside with a decreasing amount of Raji respectively. This mixed cell population was then incubated with DARPin CAR-T cells targeting CD4 or scFv CAR-T cells, targeting CD30, at an effector:(target+non-target) ratio of 1:8.

Figure 24: Anti-CD4 DARPin CAR-T cells were capable of depleting a significant amount of HuT78 target cells at all target:non-target ratios. When the target cell population consisted of 50% HuT78 and 50% Raji cells, anti-CD4 DARPin CAR-T cells depleted a mean of 61.5%

HuT78 and 14.7% of Raji cells, compared to the mock control. For anti-CD30scFv, cytotoxicity was similar (63.1% of HuT78 and 8.9% of Raji). Most efficient depletion was determined when the target population consisted of 12.5% HuT78. In this case, 73.6% of HuT78 were depleted by anti-CD4 Pin CAR-T but also 20.1% of Raji. Anti-CD30scFv CARs also had the highest mean cytotoxicity at 12.5% HuT78 (69.8%). However, depletion of Raji non-target cells reached only 5.1%. When the proportion of HuT78 cells was decreased to 0.78%, anti-CD4-DARPin CAR-Ts were still able to achieve 38.6% cytotoxicity towards HuT78, along with 10.3% of Raji. Cytotoxicity of anti-CD30scFv CAR-Ts resulted in 35.9% for HuT78 and 3.9% for Raji.

At all tested cell ratios, cytotoxicity of anti-CD4-DARPin CAR-T cells towards HuT78 target cells could be determined. Some cytotoxicity was also observed towards antigen-negative Raji cells, but at much lower levels compared to HuT78.

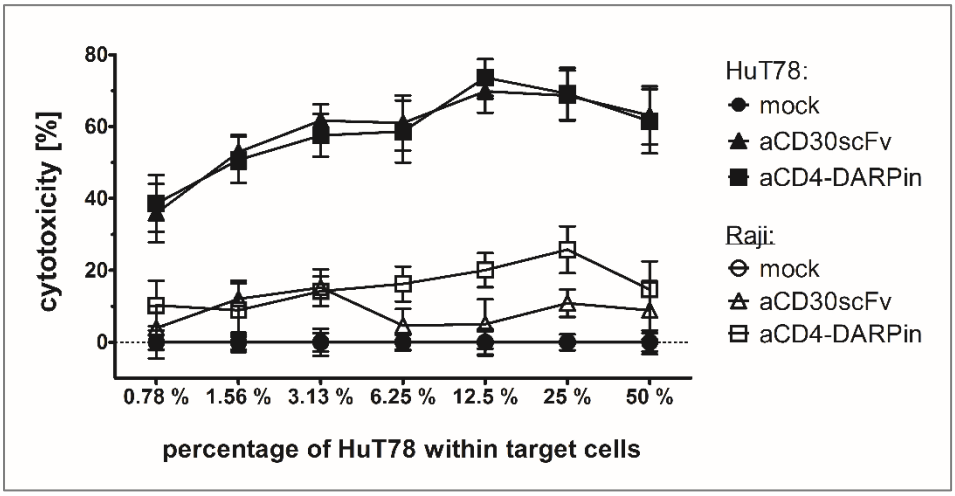

Figure 24: Specific depletion of CD4⁺ target cells in a mixed population with non-target cells.

The total number of T cells per sample remained constant at a ratio of effector cells (target + non-target) of 1:8. The number of HuT78 cells within the target/non-target mix was 50% to 0.78%. Error bars represent SEM. Assay was performed with blood from n = 6 donors in biological duplicates.

The data indicated that in mixed cell populations anti-CD4-DARPin CAR-T cells specifically depleted their relevant target cells, even if their presence was reduced to a diminutive value.

3.3 Anti-CD4-DARPin CAR-T cells deplete HIV$^+$ cells

After confirmation of specificity and potency towards an antigen-positive cell line, the next step was to investigate efficacy against HIV$^+$ target cells. Section 3.3 of this thesis describes specific activation and cytotoxicity of anti-CD4-DARPin CAR-T cells in an HIV latency model during latent as well as activated state. It was focused on the question, to what extend anti-CD4-DARPin CAR-T cells would be capable of eliminating the latent HIV reservoir and if infected cells would be depleted in an activated and latent state.

3.3.1 Depletion of CD4$^+$ T cells in a HIV$^+$ latency model

Because of the low survival rate of primary T cells after infection with HIV *in vitro*, an established latency model was used for the following assays. J-Lat cells are based on Jurkat, a cell line generated from acute T cell leukaemia. Jurkat was infected with a full-length HIV-R7 strain in which nef was substituted by GFP and a frameshift mutation in env. Hence, J-Lat cells secrete particles with incomplete capsids. The HIV genome is transcribed upon J-Lat activation and the translation into viral proteins can be detected trough expression of GFP.

Latency reversing agents induce HIV expression by J-Lat cells

When maintained under regular culture conditions, J-Lat cells are in a latent state, not expressing detectable amounts of HIV. Upon treatment with latency-reversing agents (LRAs), J-Lat start expressing HIV-GFP. Two LRAs, published to induce HIV expression in J-Lat are Prostatin and SAHA (Venkatachari *et al.* 2015). To determine the best conditions for reactivation of J-Lat, they were treated with Prostatin and SAHA at different concentrations for 24h and 48h. To see whether IFN-γ, which is secreted by CAR-T cells would influence J-Lat reactivation, it was included in the assay. Cells were analysed by flow cytometry. Reactivation was determined by expression of GFP and survival by using a live/dead cell dye (APC-eFlour). All samples were measured in duplicates. The results of this titration are illustrated in Figure 25.

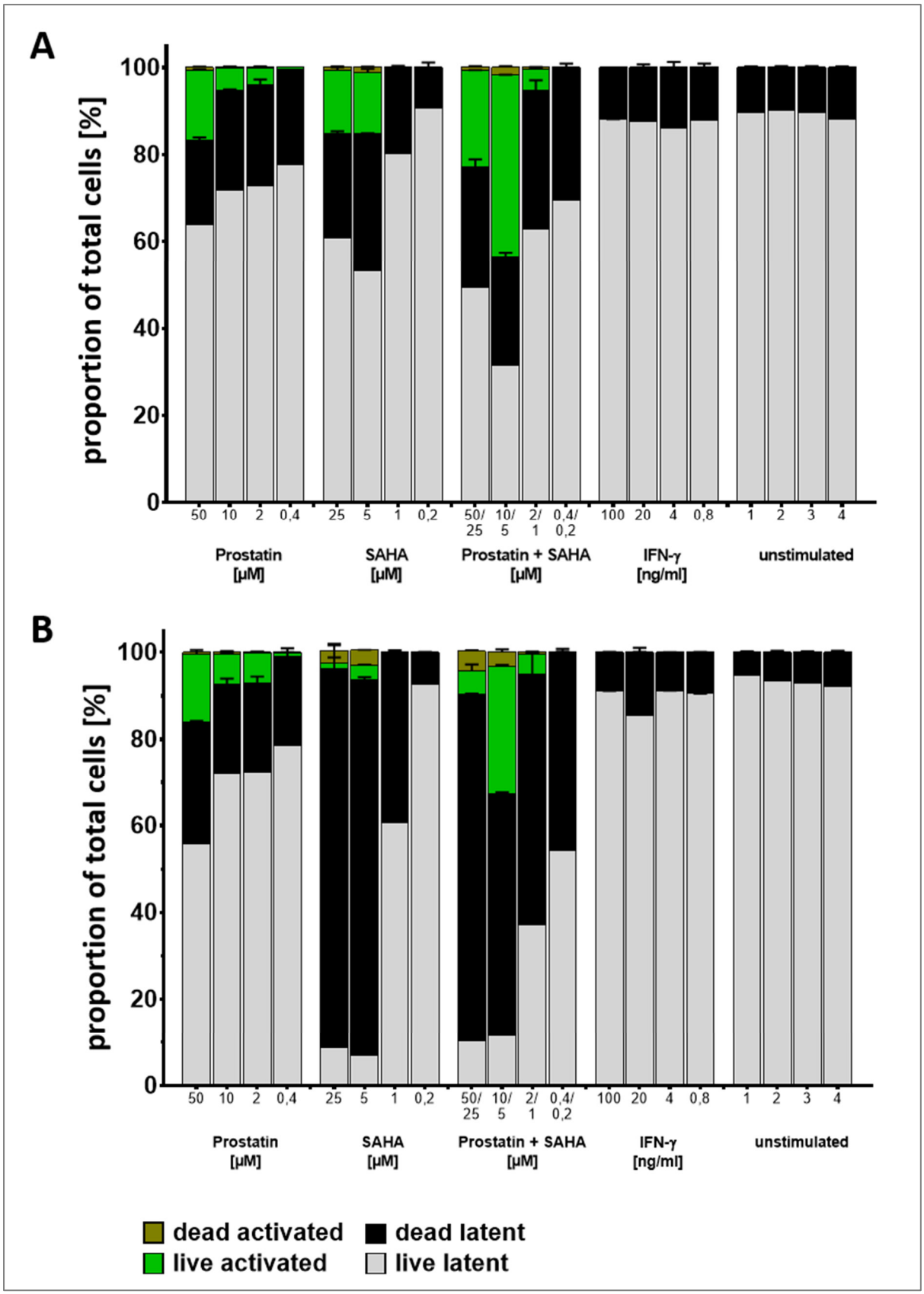

Figure 25: Titration of latency reversing agents on J-Lat cells.

Proportion of live and dead J-Lat cells expressing HIV after treatment with various doses of Prostatin and SAHA. **(A)** 24h and **(B)** 48h. Error bars represent standard error of the mean (SEM), n = 2.

In Figure 25, the survival rate of J-Lat cells after 24h was very similar between IFN-γ and untreated cells. With a range between 88.2% and 90.1%, untreated cells had only a slightly higher viability compared to IFN-γ treatment (86% to 88.1%). The same was seen after 48h, at which untreated cells were 92.2% to 94.6% viable and IFN-γ samples 85.5% to 91.2%. None of the untreated or IFN-γ treated cells expressed HIV-GFP. Overall, neither Prostatin nor SAHA could achieve high rates of J-Lat reactivation when used alone. The highest GFP expression in live cells was detected using 50 µM Prostatin: 16.1% after 24h and 15.5% after 48h. The viability decreased slightly to 70% after 48h compared to 24h with 80%-75%. However, SAHA alone did not achieve any reactivation at 0.2 and 1 µM but 14% at 5 and 25 µM after 24h. However, after 48h the majority of these samples (87%) consisted of dead cells. The highest amounts of live and reactivated cells were observed during treatment with a combination of Prostatin and SAHA. After 24h, 41.8% of cells showed to be viable and GFP^+ when treated with 10 µM Prostatin and 5 µM SAHA. In this case, 25% of cells were dead. When increasing the concentrations to 50/25 µM, reactivation was only 22.3%. After 48h, the number of reactivated cells had decreased to a maximum of 29.3%, again with 10/5 µM Prostatin/SAHA. However, viability decreased to 40.82%.

To achieve the most reasonable compromise between high HIV reactivation and low cell mortality, following assays were performed using 10 µM Prostatin in combination with 5 µM SAHA for 36h.

Depletion of latently infected J-Lat cells by anti-CD4-DARPin CAR-T cells

After the conditions for latency reversal have been optimised, a killing assay was performed in the same setup as for HuT78 (Section 3.2.1). With this assay, several questions should be answered: Will CAR-T cells be activated during co-culture with J-Lat cells? Will CAR-T cells be capable to deplete J-Lat cells? In comparison to HuT78 cells, J-Lat cells expressed low levels of CD4 (Supplementary Figure 2). Will antigen expression be sufficient to eliminate HIV^+ J-Lat cells and to what extend will the latent HIV reservoir be reactivated.

In order to not interfere with GFP expression of integrated HIV, J-Lat cells were labelled with a violet fluorescent membrane dye before incubated with CAR-T cells for 48h. Supernatant was again analysed for secretion of IFN-γ to determine CAR-specific T cell cytotoxicity, the amount of remaining J-Lat cells was determined by flow cytometry. The assay was again performed three times with two donors each. All co-cultures were prepared in duplicates of which one was analysed after 48h. In the second replicate, supernatant was replaced by medium containing Prostatin and SAHA leading to final concentrations of 10 µM and 5 µM respectively.

After additional 36h of incubation, the co-culture was analysed for amount of remaining J-Lat cells in total and J-Lat cells expressing HIV-GFP.

Figure 26 shows the results of specific CAR-T cell activation (A), cytotoxicity after 48h (B), cytotoxicity after additional 36h of reactivation with LRAs (C) and HIV expression after treatment with LRAs (D).

Activation of CAR-T cells by J-Lat cells (Figure 26 A) generally resulted in lower IFN-γ levels than by HuT78. The highest IFN-γ levels for anti-CD4-DARPin CAR-T cells were detected at an effector:target ratio of 1:8 with 0.13 ng/ml. Control CARanti-CD30scFv induced 0.209 ng/ml at 1:8 (E:T). With decreasing E:T ratios, IFN-γ decreased as well. For anti-CD4-DARPin CAR-T cells, IFN-γ was released to 0.046 ng/ml at E:T 1:32, this value had decreased to 0.007 ng/ml and below 1:256, IFN-γ was below detection and the highest background was produced by mock CAR-T cells only at 0.016 ng/ml. Figure 26 B: After 48h of co-culture, anti-CD4-DARPin showed highest specific cytotoxicity of 84.78% at 1:8 (E:T). At 1:64 (E:T), cytotoxicity towards $CD4^+$ J-Lat cells was still 56.04%, at 1:256 at 26.07% and at the lowest effector:target ratio, CD4-specific cytotoxicity was at 6.4%. similar values, between 89.26% and 1.75% were detected for annti-CD30scFv control CAR.

In the second co-culture replicate, supernatant was replaced by fresh medium containing LRAs (10 µM Prostatin and 5 µM SAHA). After 36h of incubation with LRAs, survival and HIV-expression of membrane-labelled J-Lat cells were analysed in flow cytometry (Figure 26 C). Cytotoxicity was very similar to 48h (Figure 26 B). Cytotoxicity of anti-CD4-DARPin CAR-T cells started at 5.04% at 1:1024. It then decreased in a dose-dependent manner to 59.59% at 1:64 (E:T) and 79.53% at 1:32 (E:T). Cytotoxicity slightly increased further to the peak of 88.93% at 1:16 (E:T). At the highest effector:target ratio of 1:8, cytotoxicity was with 86.93% slightly lower. Dose-dependent cytotoxicity of control CAR-T cells anti-CD30scFv was very similar to anti-CD4-DARPin. The values found started at 2.35% (1:1024, E:T) and ended at 86.98% (1:8, E:T) with a peak at 89.96% (1:16, E:T).

In addition to specific killing of J-Lat cells before and after treatment with LRAs, expression of HIV was determined in comparison to mock control T cells (Figure 26 D). After co-culture with anti-CD4-DARPin CAR-T cells (black columns) and treatment with LRAs, 90.64% of J-Lat cells were reactivated to express HIV-GFP compared to mock control (light grey columns) at 1:1024 (E:T). HIV-GFP expression was decreased alongside with increasing effector:target ratios: 80.7% (1:512), 56.34% (1:256), 37.53% (1:128), 22.25% (1:64), 10.55% (1:32). The lowest reactivation of HIV was achieved at 1:16 (E:T), only 5.43% of J-Lat cells expressed HIV, compared to mock control. When the effector:target ratio was then increased to 1:8, J-Lat reactivation was slightly higher again (12.24%). This correlates with the observation that also the cytotoxicity towards J-Lat cells at this ratio was lower compared to 1:16 (Figure 26 C). Control

scFv-CAR-T cells targeting CD30 did achieve similar results. In this case, HIV reactivation started at 94.4% (1:1024, E:T) and then also decreased with increasing effector:target ratios: 90.61% (1:512), 75.74% (1:256), 58.25% (1:128), 27.35% (1:64), 16.58% (1:32). For anti-CD30scFv control CAR, HIV-GFP expression was also the lowest (3.34%) at 1:16 (E:T)and increased slightly to 7.85% at 1:8. This observation also correlates with the fact that the cytotoxicity towards J-Lat cells had been decreased at this ratio (Figure 26 C). Figure 27 shows expression of HIV-GFP by reactivated J-Lat cells after co-culture with CAR-T cells in fluorescent microscopy. The images were produced for 4 representative donors at effector:target ratios of 1:8 and 1:256. A clear reduction of GFP-expression can be seen after co-culture with anti-CD4-DARPin CAR-T cells, in comparison to mock T cells. This effect is reduced at lower effector:target ratios (1:256).

This experiment showed that J-Lat cells do induce activation and secretion of IFN-γ of anti-CD4-DARPin CAR-T cells. The overall values were lower compared to HuT78 target cells but the dose-dependent pattern was the same. The low expression of target antigen also showed to be sufficient to induce specific cytotoxicity of anti-CD4-DARPin CAR-T cells towards J-Lat cells. Hence, reactivation of J-Lat cells for production of HIV-GFP was strongly decreased.

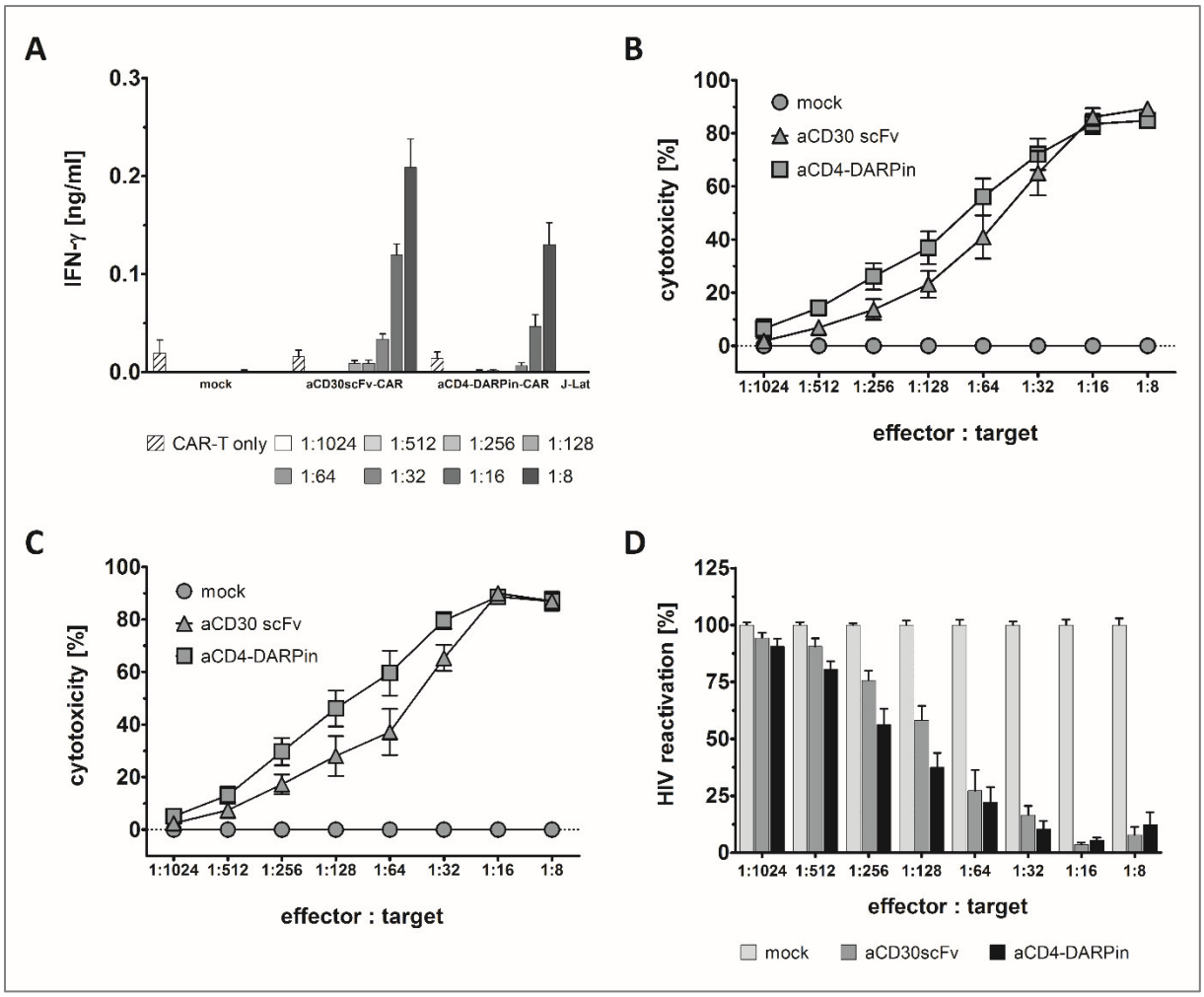

Figure 26: Dose-dependent depletion of HIV⁺ J-Lat cells.

(A) CAR-T cell activation was determined by recording IFN-γ in the supernatant after 48h co-incubation with J-Lat cells. **(B)** Number of remaining J-Lat cells after 48h was determined by flow cytometry. Cytotoxicity [%] was specified as reduction of target cells compared to respective mock control. **(C, D)** After 48h of co-cultivation, cells were activated with Prostatin and SAHA for additional 36h to induce the expression of HIV-eGFP. Total number of J-Lat cells was determined by flow cytometry **(C)**, as well as the number of activated HIV-eGFP expressing cells **(D)**. Assays were performed in biological duplicates with cells from n = 6 donors. Error bars represent standard error of the mean (SEM).

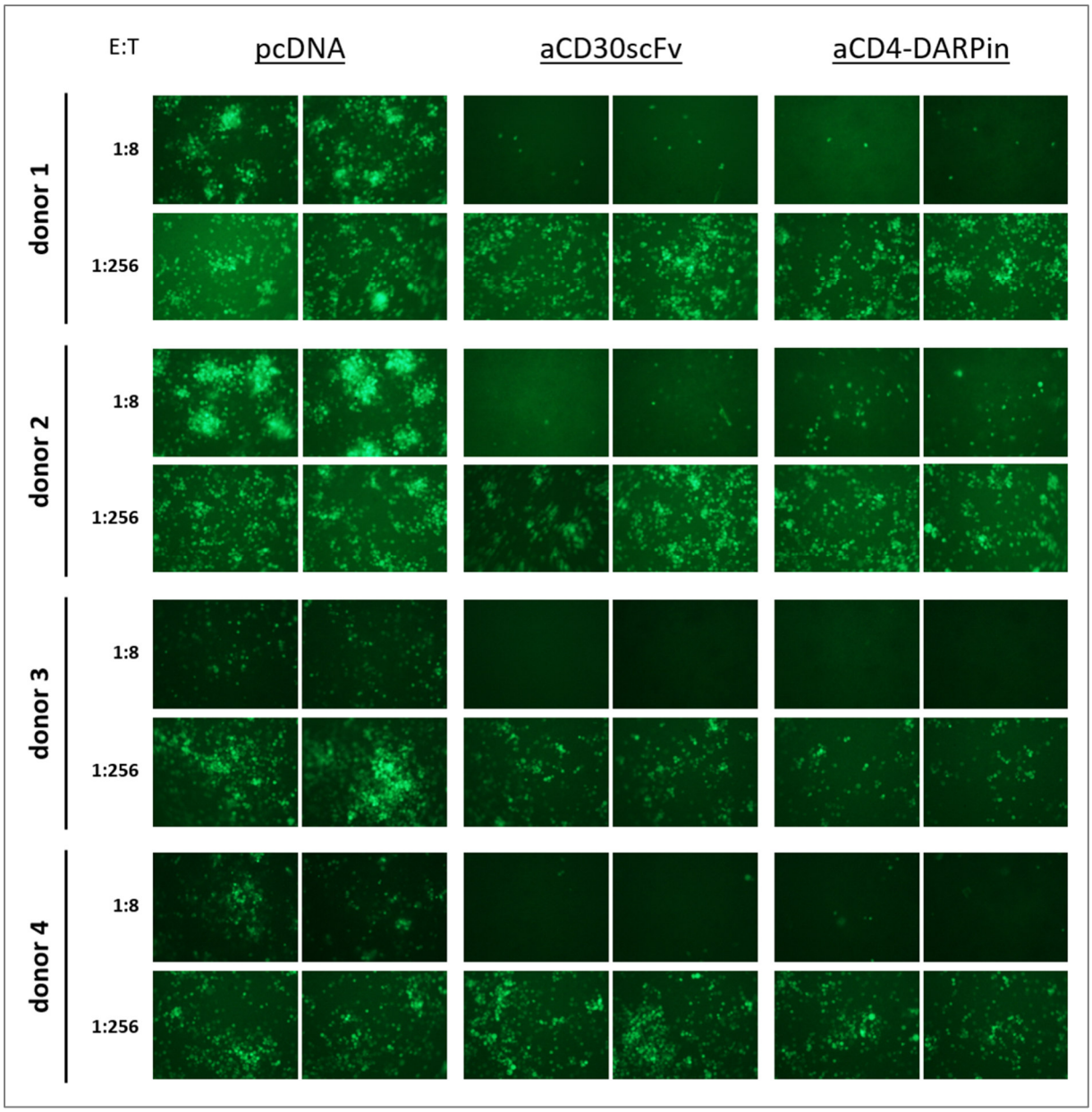

Figure 27: Expression of HIV-eGFP by J-Lat cells after co-culture with CAR-T cells

After 48h co-culture with CAR-T cells, J-Lat cells were activated with Prostatin and SAHA for additional 36h. Expression of HIV-eGFP is shown by fluorescent microscopy for 4 donors in duplicates at exemplary effector:target ratios of 1:8 and 1:256. (100x, 250ms)

Depletion of activated HIV$^+$ J-Lat cells by anti-CD4-DARPin CAR-T cells

Reflecting on a possible treatment of HIV-infected patients with CAR-T cells, the majority of CD4$^+$ T cells is going to be in a latent state, especially after extensive cART. But it is possible that some target cells are activated and expressing HIV to some extent. Furthermore, the release of cytokines such as IFN-γ is likely to induce reactivation of latently infected cells. Therefore, it should be tested, whether anti-CD4-DARPin CAR-T cells are also capable of depleting activated J-Lat cells after treatment with LRAs.

In this experiment, J-Lat cells were treated with 10 µM Prostatin and 5 µM SAHA for 36h before co-culture with CAR-T cells for 48h. Cells were then again analysed for amount of remaining J-Lat cells in general and J-Lat cells expressing HIV-GFP by flow cytometry. It was shown previously, that treatment with LRAs alone already decreases viability of J-Lat cells (Figure 27). Co-culture with large numbers of primary T cells in the same well would impact the survival even more, due to lack of nutrition and unspecific cell-cell interactions. Therefore, effector:target ratios had to be reduced from 1:32 to 1:4096 in order to still have a sufficient number of surviving J-Lat cells in the mock control samples.

Figure 28 A shows the specific cytotoxicity of anti-CD4-DARPin and anti-CD30scFv CAR-T cells compared to mock control. While at the lowest anti-CD4-DARPin CAR-T effector:target ratio of 1:4096 specific cytotoxicity was insignificantly low (0.27%), it was increased with higher CAR-T cell doses: 7.9% (1:2048), 9.9% (1:1024), 14.11% (1:512), 31.92% (1:256), 62.54% (1:128), 85.37% (1:64). The highest value for specific cytotoxicity of anti-CD4-DARPin CAR-T cells towards activated J-Lat cells was obtained at the highest T cell dose: 92.14% specific cytotoxicity at 1:32 (E:T). Control CAR-T cells expressing anti-CD30scFv, showed again similar cytotoxicity: 0.94% (1:4096), -2.66% (1:2048), 3.2% (1:1024), 14.8% (1:512), 38.97% (1:256), 73.8% (1:128), 95.5% (1:64) and 96.43% (1:32). The amount of remaining J-Lat cells expressing HIV-GFP after CAR-T co-culture is shown in Figure 28 B. While anti-CD4-DARPin CAR-T cells had no impact at the lowest effector:target ratio of 1:4096, 100.1% compared to mock T cells, remaining activated J-Lat cells could be decreased along with increasing CAR-T cell ratios: 92.7% (1:2048), 89.57% (1:1024), 86.7% (1:512), 74.98% (1:256), 47.2% (1:128) and 26.85% (1:64). At the highest ratio of 1:32, the amount of HIV-expressing target cells was decreased to 19.87% compared to mock. Again, results obtained for anti-CD4-DARPin correlated with those for anti-CD30scFv control CAR: 101.47% (1:4096), 104.64% (1:2048), 94.28% (1:1024), 86.59% (1:512), 64.23% (1:256), 35.53% (1:128), 11.69% (1:64) and 10.62% (1:32).

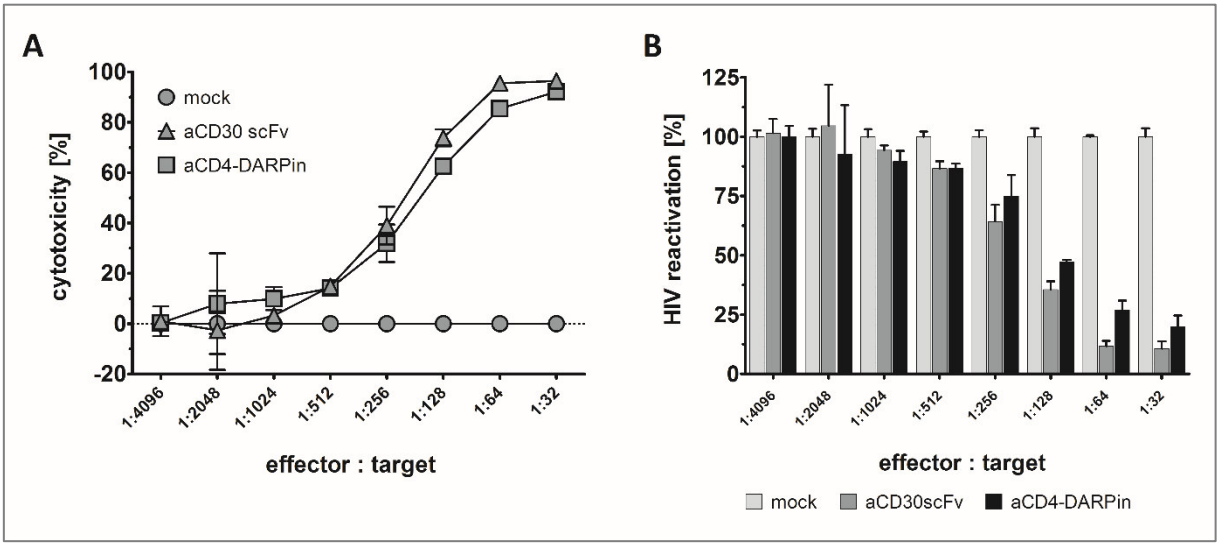

Figure 28: Dose-dependent depletion of activated HIV⁺ J-Lat cells.

(A) Cytotoxicity [%] of J-Lat cells in total, after pre-activation for 36h followed by 48h co-culture with CAR-T cells. **(B)** Remaining activated J-Lat cells after 36h activation and 48h co-culture. Assays were performed in biological duplicates with cells from n = 2 donors. Error bars represent standard error of the mean (SEM).

Anti-CD4-DARPin CAR-T cells showed specific cytotoxicity towards reactivated J-Lat cells and reduced HIV expressing cells below 20% compared to mock T cells.

3.4 Specific depletion of autologous CD4⁺ cells by DARPin CAR-T cells

During CAR-T cell therapy, T cells are isolated from the patient's blood and transduced with the respective CAR vector particles. The CAR-T cells are then transfused back into the patient where they target tumour-associated self-antigens. Thus, CAR-T cells induce autologous cytotoxicity. To evaluate whether anti-CD4-DARPin CAR-T cells would be capable to specifically deplete CD4⁺ T cells of their own donor, a killing assay was performed using autologous primary T cells. In this setup, T cells were transduced with anti-CD4-DARPin CAR without prior magnetic depletion of CD4⁺ T cells. The population therefore consisted of CD4⁺ and CD8⁺ cells which was monitored by flow cytometry over 8 days. The population was tested for expression of T cell marker CD3 and subpopulations expressing CD4 and CD8 before transduction and on days 3, 5 and 8 (Figure 29).

Immediately prior to transduction, all samples contained around one half of CD4⁺ cells: mock T cells 49.84%, anti-CD30scFv 52.05%, anti-CD4-DARPin 49.84%. On day 3 post transduction, both mock and anti-CD30scFv CAR-T cells reduced their CD4 T cell ratio slightly to 44.14% and 41.04% respectively. Within the samples transduced with anti-CD4-DARPin CAR, the T cell populations had already shifted towards a mean of 22.28% CD4⁺ and 64.77% CD8⁺.

This trend continued on days 5 and 8, when mock control consisted of a mean of 40.38%/34.78% $CD4^+$ along with 51.77%/56.83% $CD8^+$ cells. Anti-CD30scFv CAR T cells reduced their $CD4^+$ T cell faction from 36.73% on day 5 (55.38% $CD8^+$) to 29.94% on day 8 (62.16% $CD8^+$). In contrast, samples of anti-CD4-DARPin CAR-T cells decreased the $CD4^+$ proportion much faster resulting in 6.9% on day 5 and 1.22% on day 8. The population of $CD8^+$ T cells therefore increased to 79.88% on day 5 and on day 8, 87.3% of the T cell population was $CD8^+$. Double-positive cells expressing CD4 and CD8 were negligible. Double-negative T cells, expressing neither CD4 nor CD8 remained at levels below or slightly above 10% during 8 days post transduction. It cannot be determined, whether these populations were really double-positive/-negative or if this was an effect of the staining with fluorescently labelled antibodies.

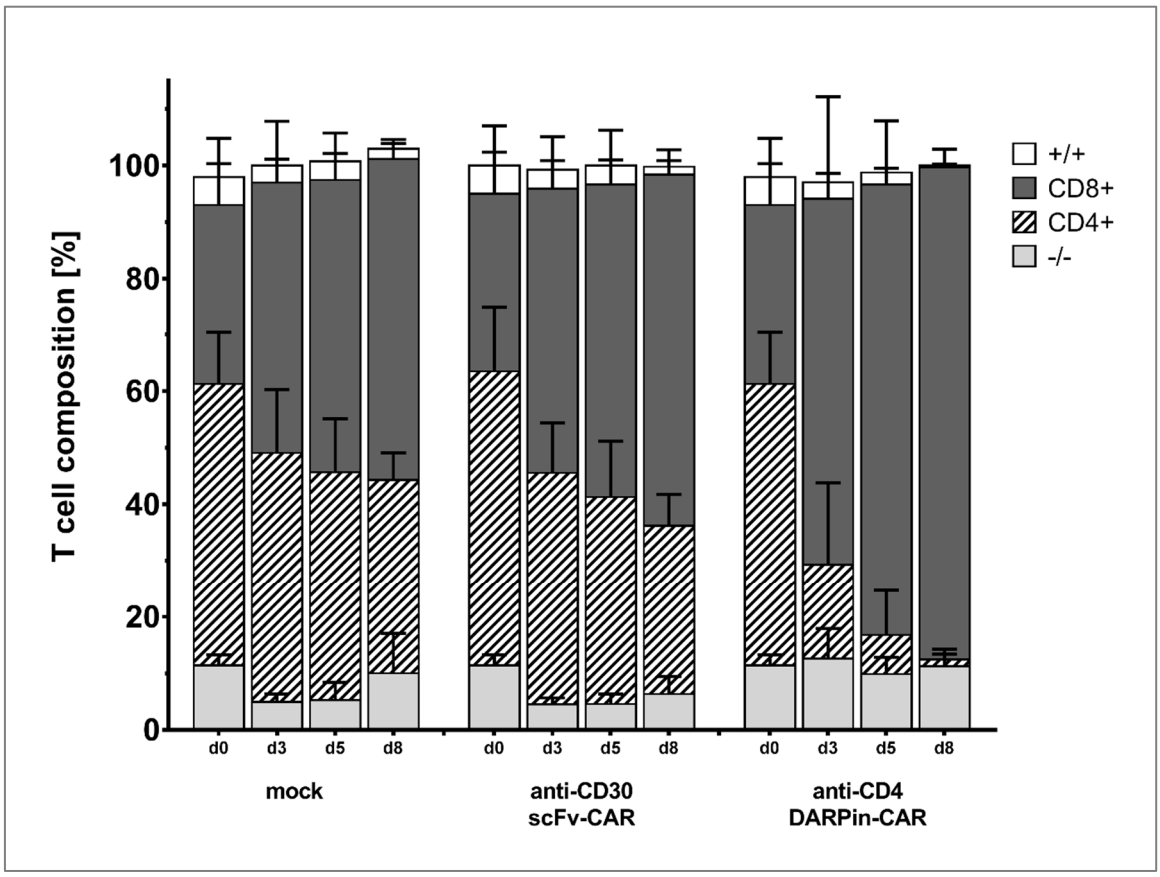

Figure 29: Killing of autologous $CD4^+$ T cells.

Composition of T cell subsets from peripheral blood after incubation with autologous anti-CD4-DARPin-CAR-T cells. $CD3^+$ T cells from 6 donors were analysed by flow cytometry for the expression of CD4 (diagonal pattern) and CD8 (dark grey) on days 0, 3, 5 and 8 post transduction with MLV-based vectors encoding the anti-CD30scFv-CAR or anti-CD4-DARPin-CAR or with empty vectors (mock). Data represent the standard deviation of the mean (SD), n = 6 donors.

Anti-CD4-DARPin CAR-T cells were capable to specifically deplete autologous $CD4^+$ T cells to a mean of 1.22%.

3.5 Generation of non-human primate CAR-T cells

Depletion of HIV infected cells using CD4-targeted CAR-T cells requires extensive testing and investigation. The evaluation of efficacy and safety of this approach will have to be tested within an immunocompetent animal model. Before applying this approach into a clinical trial, essential questions must be investigated. As for all CAR-T cell targets, the specificity towards the diseased tissue needs to be validated. CD4 is exclusively expressed in bone lymphoid cells (The Human Protein Atlas 2018). It is a requirement that CAR-T cells do not bind or damage any healthy tissues. In case of CD4 targeting HIV infected cells, efficacy will have to be proven in an in vivo latency model. Will all infected cells be eliminated so that no viral particles are produced in absence of cART? What other effects are caused by anti-CD4 T cells within the immune system? Will the CD4 T cell population recover after CAR-T cell treatment? Because HIV and SIV exclusively infect their natural hosts, humans and non-human primates, rodent animal models are only suitable to answer particular questions. In order to mimic the situation in an immunocompetent animal model, anti-CD4 CAR-T cells will have to be tested in non-human primates – which are currently the only animal model for HIV/SIV (Chen 2018). The most established animal model for HIV infection are rhesus (*Macaca mulatta*) and *African Green Monkeys* (*Chlorocebus*). Infection of rhesus monkeys with SIV has the same progression of disease as HIV infection in humans, leading to AIDS if untreated. *African Green Monkeys* (AGMs) in turn are natural hosts for SIV. SIV in AGMs remains in a latent state without any treatment. These animals do not develop disease symptoms and viral loads usually remain below detection level without latency reversion. Rhesus and AGM are therefore the most suitable animal models to investigate physiological effects and efficacy of anti-CD4-DARPin CAR-T cells during HIV/SIV infection.

3.5.1 Design and generation of non-human primate derived CARs

Intracellular CAR domains need to be able to activate the T cell they are expressed on. CAR-T cells are derived from autologous (patient-derived) PBMCs and the CAR activation domain has been derived from human CD28 and CD3zeta receptors chains. These receptor chains must interact with the intracellular kinases and other enzymes to initiate intracellular signalling pathways (Section 1.1.3). CD28 and CD3zeta chains of humans and non-human primates are not 100% equal (Figure 30). Homology between the CAR activation domains of humans and rhesus monkeys is 95.63%, between rhesus and AGM 97.81% and 95.10% between humans and AGMs (Figure 30B). For the design of non-human primate CARs, the intracellular activation domain was therefore adjusted to respective sequences of rhesus and AGM. Figure 30A shows the alignment of CAR activation domains of human, rhesus and AGM. Heterologous

peptides are highlighted with red asterisks and ITAM domains with a yellow background. The Lck phosphorylation site within the CD28 co-activation domain is shown in the wild-type format. The CARs used in this thesis have a depleted DLck binding domain, which inhibits Lck phosphorylation. The rationale behind this is that the Lck pathway leads to secretion of IL-2, which supports survival and proliferation of T_{reg} cells. T_{reg} cells suppress the immune response of cytotoxic CD8 T cells, which reduces CAR-T efficacy of CAR-T cells. By depleting the Lck pathway, signalling of the PI3K pathway is enhanced, resulting in increased IFN-γ release which in turn promotes T cell activation (Kofler *et al.* 2011).

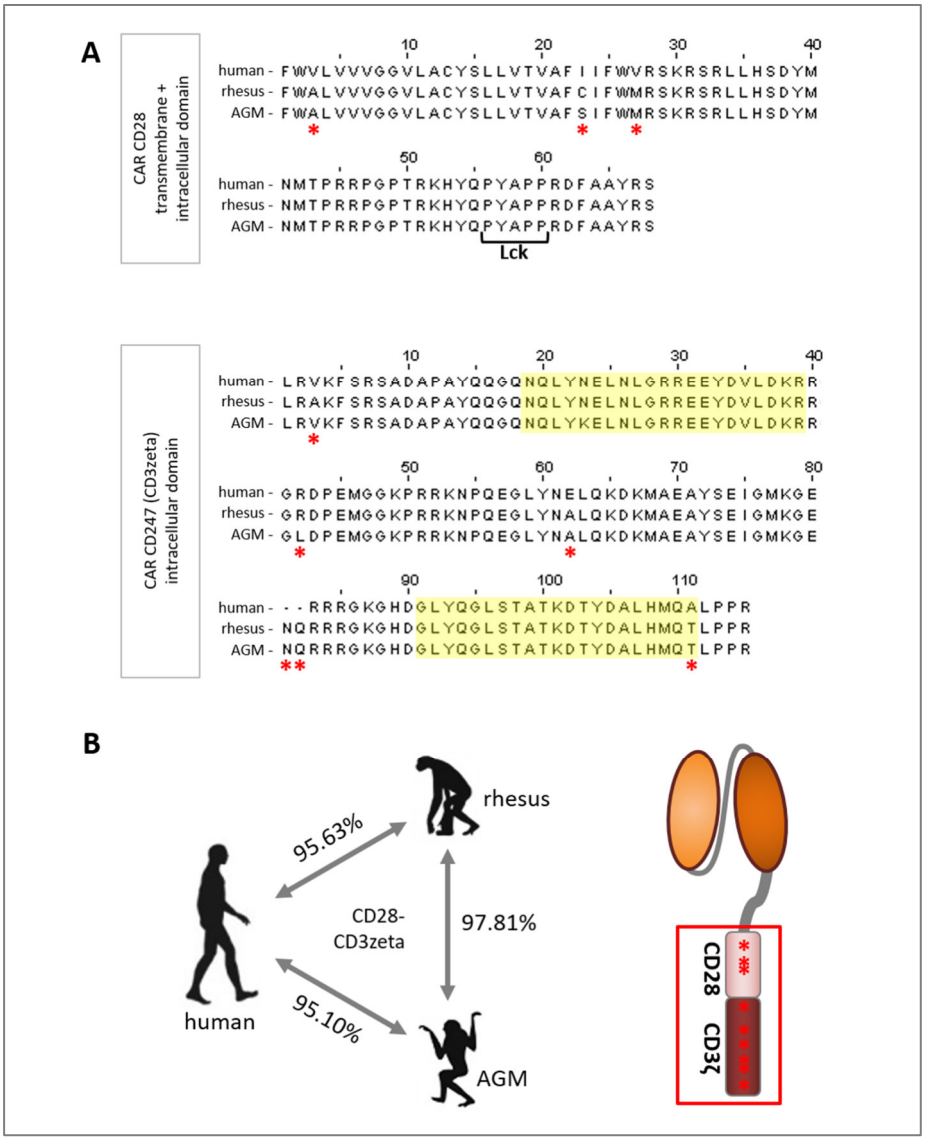

Figure 30: Species specific CAR activation domains

Alignment of CAR activation domains CD3zeta (CD247) and CD28 with a wild-type Lck domain. **(A)** Alignment of peptide sequences between human (as in plasmid #11833 pBullet), rhesus (*Macaca mulatta*: CD28 NP_001036106.2; CD247 NM_001077423.1) and AGM (*Chlorocebus sabaeus*: CD28 X2: XM_007965942.1; CD247_X2, NCBI XM_007989658.1). Divergent amino acids are marked with red asterisk. **(B)** Sequence homology between human, rhesus and AGM CAR domains and respective domains within the CAR. (NCBI 2018)

Non-human primate derived CAR activation domains were designed in silico and then ordered in a subcloning plasmid from GeneArt (Invitrogen). The domains were exchanged via *MluI* and *SbfI* restriction sites. To deplete the Lck binding domain (PYAPP) into DLck (AYAAA), a mutagenesis PCR was performed as described in Section 2.2.1. Insertion of the nhp domain was confirmed by GATC sanger sequencing. Transfer vector plasmids encoding for all CAR variants were generated: anti-CD30scFv and anti-CD4 DARPin binding domains in combination with CD28/CD3zeta activation domains derived from human, rhesus and AGM. For each construct, one variant containing the wild type Lck domain and the depleted DLck domain was cloned.

GaLV pseudotyped gamma-retroviral particles encoding for non-human primate CARs were produced as described in Section 0 and detected by MLV gag-protein p30 in Western blot (Section 2.2.3). The western blot in Figure 31 shows detection of human and nhp derived retroviral particles by MLV gag p30. All particles show similar levels of expression, as well as the mCherry control CAR. A slightly lower number of particles was detected in the mock control (lane1), which contained pcDNA instead of a CAR transfer vector plasmid. No p30 protein was detected blotting supernatant of untransfected HEK293T cells.

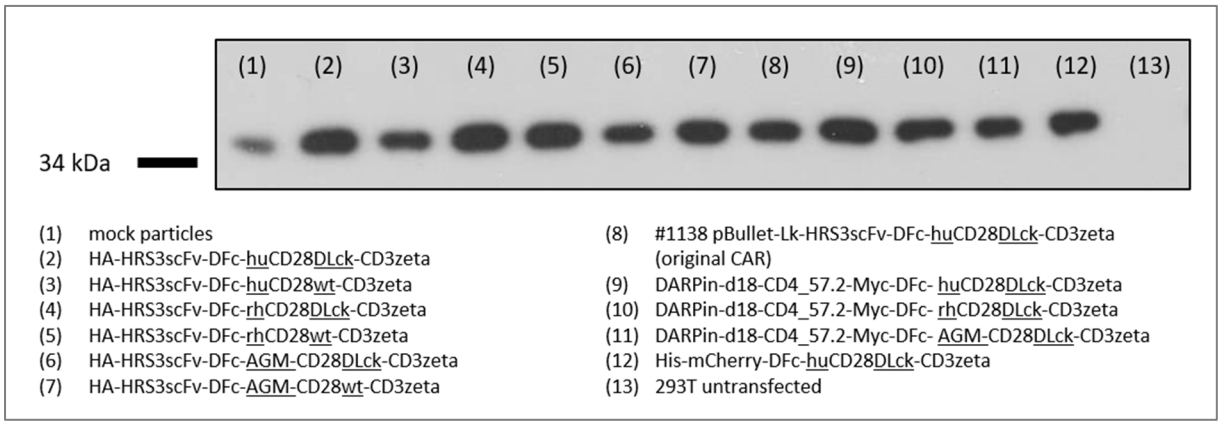

Figure 31: Detection of CAR encoding gamma-retroviral particles by Western Blot.

Expression of MLV-gag protein p30 by γ-retroviral vectors in supernatant of transfected HEK293T/17 cells. Particles used for T cell co-culture assays: empty mock control (lane2) or CAR delivering vector particles (lanes 2-12). CARs contained binding domains anti-CD30-HRS3scFv or anti-CD4-DARPin_57.2. CAR activation domains were derived from human, rhesus or AGM receptors CD28-CD3zeta, containing wild-type Lck or a depleted DLck domain. Supernatant of untransfected cells served as negative control (lane 13).

3.5.2 CAR expression by non-human primate T cells

To generate non-human primate CAR-T cells, PBMCs were isolated from fresh blood of rhesus macaques and AGMs (Section 2.2.5). Specific activation of nhp T cells was achieved by culture on plates coated with nhp cross-reactive anti-CD3 (clone SP34.2) and anti CD28 (clone 15E8) antibodies. After 4 days of activation, T cell purity was confirmed by staining of T cell marker CD3. T cell activation was determined by staining for T cell activation marker CD25. Figure 32 shows the expression of CD25 on T cells of one rhesus and one AGM in flow cytometry. For the rhesus macaque, 24.66% of all T cells expressed CD25 after 4 days of activation with anti-CD3 and anti-CD28 antibodies while 81.49% of AGM T cells expressed.

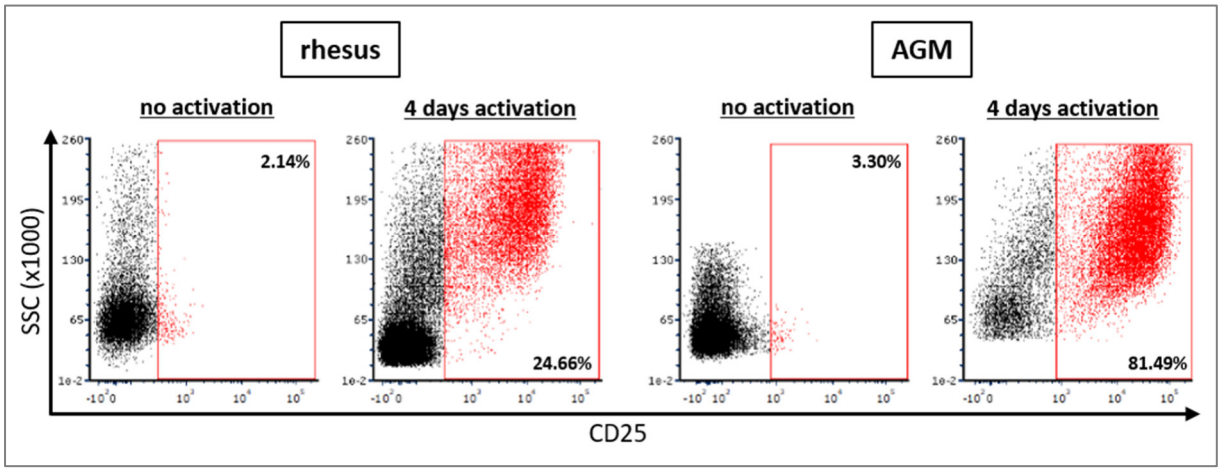

Figure 32: Specific activation of non-human primate T cells.

Expression of T cell activation marker CD25 on T cells of rhesus and AGM after 4 days without and with activation with anti-CD3 and anti-CD28 antibodies. Positive expression was assessed against the respective CD25 isotype control.

Activation of nhp PBMCs with nhp cross-reactive antibodies against CD3 and CD28 led to specific proliferation and activation.

After successful activation, non-human T cells were transduced with GaLV-pseudotyped MLV particles encoding for nhp derived CARs. Transduction followed the same protocol as human T cells (Section 43). CAR expression was assessed in T cells of two rhesus macaques and two AGMs over 8 days post transduction. T cells were transduced without magnetic depletion of CD4 T cells.

Figure 33 shows the expression of species-specific CARs by nhp T cells on days 3, 5 and 8 after transduction. CARs encoded the CD28Dlck co-stimulation domains. CAR expression was assessed by staining the IgG CAR spacer domain with a PE-labelled antibody. Fluorescent signal was determined in flow cytometry against the Fab2 isotype control. Within both rhesus macaques (Figure 33 A+B), expression of both, CD30 and CD4 specific CARs varied between

8.67% and 11.44% and did not show any clear trends of up- or downregulation within 8 days. Transduction of AGM T cells led to slightly more CAR expression than in rhesus. Three days post transduction, between 12.09% and 21.42% of AGM T cells expressed CARs (Figure 33 C+D). In AGM284, CAR expression dropped down to 3.98% for anti-CD30scFv CAR and 7.50% for anti-CD4-DARPin CAR. In AGM350, CAR expression dropped slightly less, to 9.21% for anti-CD30scFv CAR and 16.39% for anti-CD4-DARPin CAR.

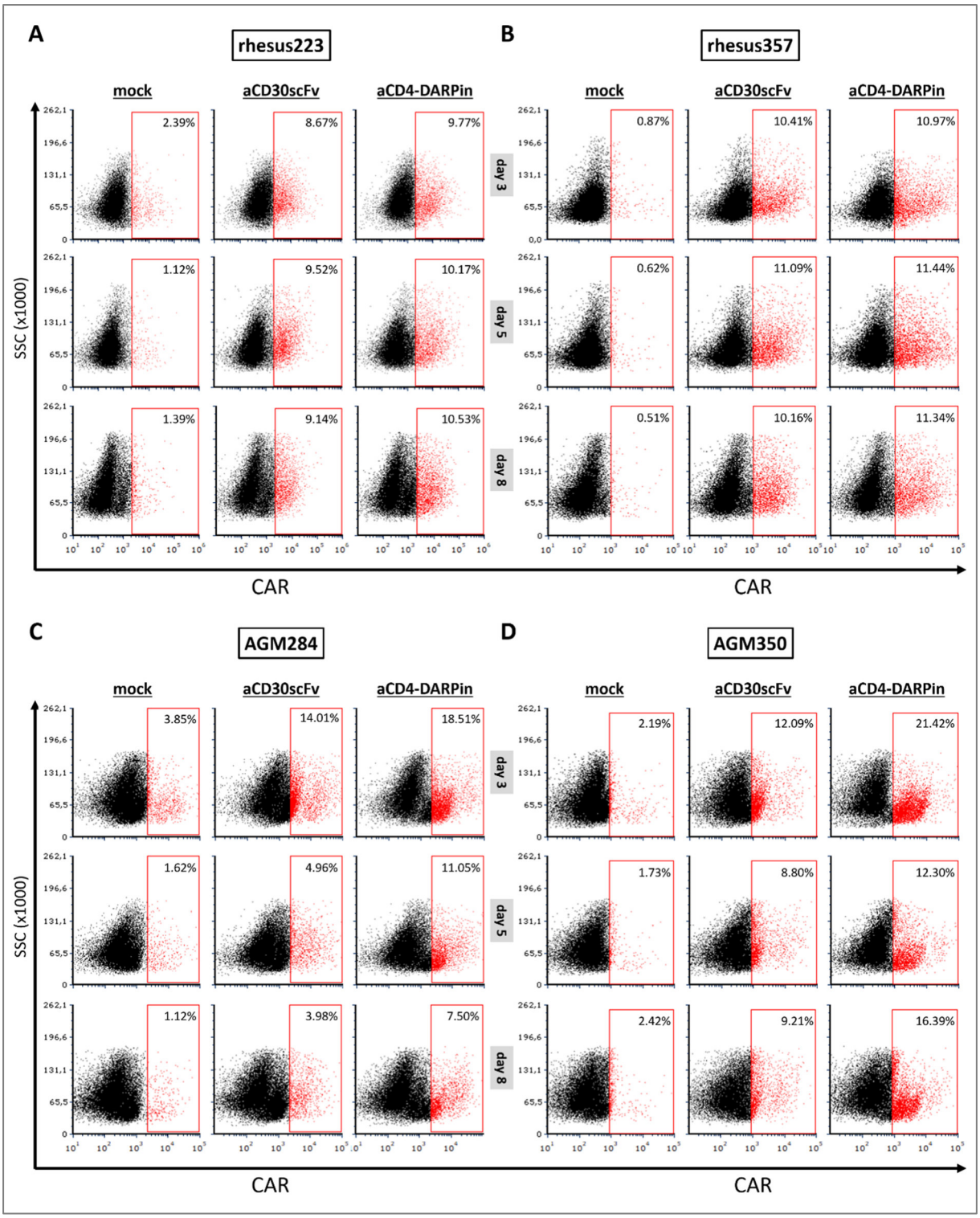

Figure 33: CAR expression by non-human primate T cells.

CAR expression of T cells of 4 non-human primates, before transduction and on days 3, 5 and 8 post transduction with gamma-retroviral particles. Viral particles encoded for mock control, anti-CD30scFv-CAR or anti-CD4-DARPin CAR. CAR expression was assessed by flow cytometry. **(A)** resus223, **(B)** rhesus357, **(C)** AGM284, **(D)** AGM350.

T cells of rhesus macaques and AGM were successfully transduced with MLV particles, leading to expression of CARs containing species-specific activation domains. CAR expression in AGM T cells dropped but was still maintained until day 8 post transduction.

3.5.3 Functionality of non-human primate CAR-T cells

After transduction of nhp T cells with MLV particles and expression of anti-CD30scFv CARs and anti-CD4 CARs with species-specific CD28DLck-CD3zeta activation domains, they were stained for expression of T cell marker CD3, CD4 and CD8, to test functionality of the CAR. A proportion of the T cells of two rhesus macaques and two AGMs were stained before and on days 1 (except rhesus223), 3, 5 and 8 post transduction. Proportion of CD4 and CD8 expressing cells were analysed in flow cytometry. Figure 34 shows the development of CD4 and CD8 T cell populations within nhp CAR or mock transduced T cells. Samples were co-stained for CAR expression, which is shown in Figure 33. The detailed flow cytometry plots of these samples are shown in Supplementary Figure 4 (rhesus223), Supplementary Figure 5 (rhesus375), Supplementary Figure 6 (AGM284) and Supplementary Figure 7 (AGM350). T cell populations of rhesus223 and rhesus357 started before transduction from 46.04% and 54.34% $CD8^+$ along with 33.36% and 26.85% $CD4^+$. After 8 days, mock transduced T cells showed a slight shift towards the $CD8^+$ population (26.34% and 20.92% $CD4^+$). This decrease of $CD4^+$ T cells was even stronger within anti-CD30scFv expressing cells, resulting in 20.46% and 18.51% $CD4^+$ cells. The strongest decrease of $CD4^+$ T cells was observed after transduction with anti-CD4-DARPin CARs: rhesus223 65.04% $CD8^+$ and 16.41 $CD4^+$, rhesus357 74.97% $CD8^+$ and 9.57% $CD4^+$ T cells. This shift towards the $CD8^+$ population was not observed in CAR expressing AGM T cells. Both samples showed rather an increase of the $CD4^+$ population. AGM284 started with 54.34% $CD8^+$ and 29.13% $CD4^+$ T cells. Within 8 days post transduction, the mock control consisted of 57.45% $CD8^+$ and 40.72% $CD4^+$ T cells. For anti-CD30scFv CAR expressing T cells, this increase of the $CD4^+$ population was even stronger, leading to 50.78% $CD8^+$ and 48.06% $CD4^+$ T cells. This effect was similar for anti-CD4-DARPin CAR expressing cells of AGM284. Eight days post transduction, 49.33% of T cells were $CD4^+$ and 50.55% $CD8^+$. The same increase of the $CD4^+$ T cell proportion was detected in AGM350 T cells. They started with 72.80% $CD8^+$ and 24.02% $CD4^+$, before transduction. On day 8, all samples had reached nearly 50% $CD4^+$ cells: mock 50.35% $CD4^+$/49.00% $CD8^+$, anti-CD30scFv-CAR 52.01% $CD4^+$/47.05% $CD8^+$ and anti-CD4-DARPin CAR 51.77% $CD4^+$/47.71% $CD8^+$ cells. After transduction with species-specific CARs, T cells of rhesus macaques showed depletion of autologous $CD4^+$ T cells. However, this depletion was less efficient than in human T cells. For AGM T cells, an increase of the $CD4^+$ T cell population was observed.

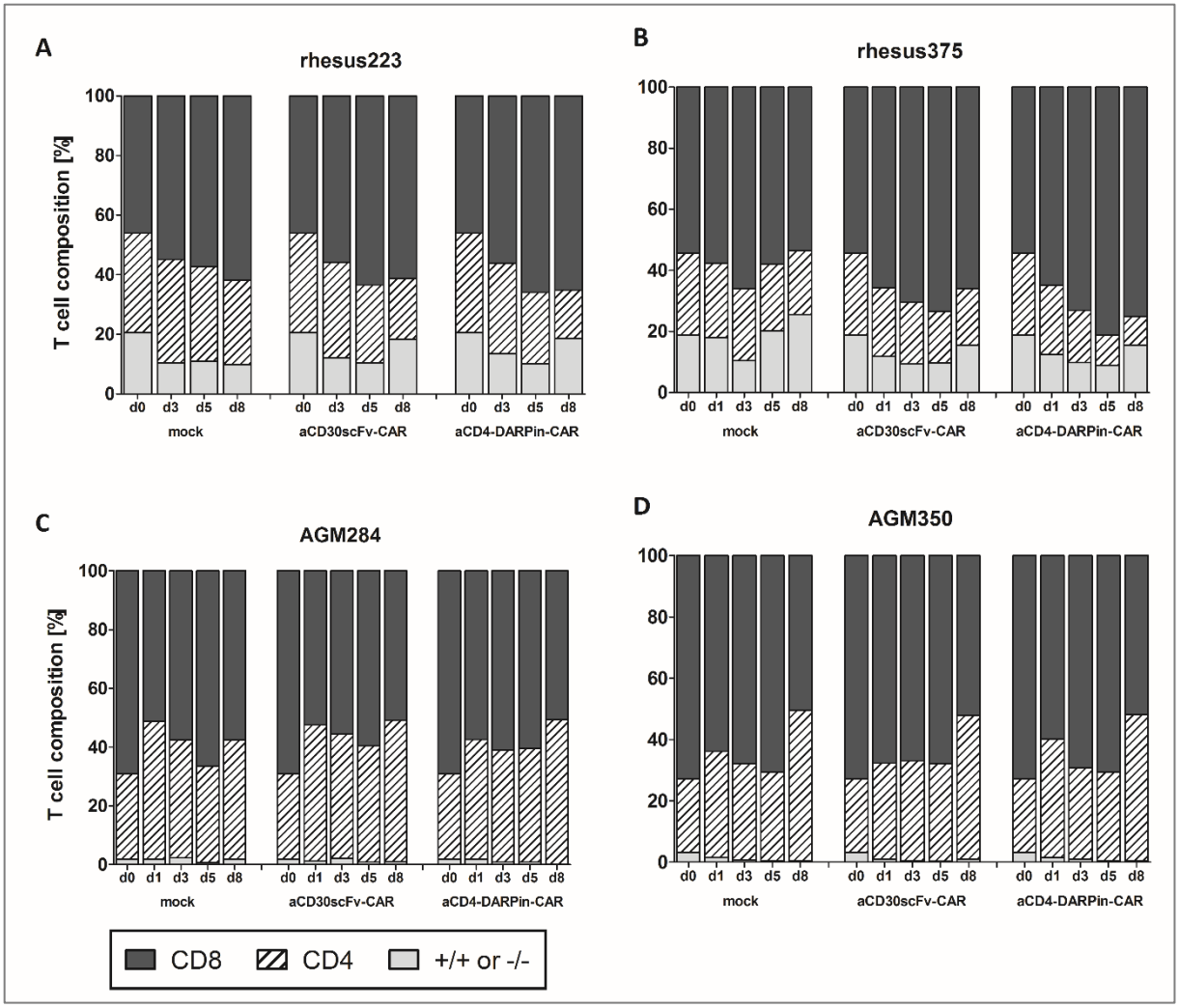

Figure 34: Monitoring of CD4 and CD8 populations in non-human primate CAR-T cells.

Proportion of CD4 and CD8 positive T cells on 4 non-human primates over time. Cells were analysed before and until day 8 after transduction with CAR or mock encoding retroviral particles. CD8 populations are shown in dark grey and CD4 populations in diagonal pattern. **(A)** resus223, **(B)** rhesus357, **(C)** AGM284, **(D)** AGM350.

3.6 Results summary

Restriction sites of single-cutting enzymes were inserted into the expression plasmid of a second-generation anti-CD30scFv CAR, to be able to exchange the different CAR domains individually. This, the anti-CD30scFv binding domain was replaced by an anti-CD4-DARPin. This expression plasmid was then co-transfected into HEK293T cells, to produce gamma-retroviral transfer-vector particles. These were then transduced into activated primary T cells. CAR-expression on the surface of human and non-human primate T cells was confirmed by flow cytometry.

Specificity and potency of the generated anti-CD4-DARPin CAR-T cells were assessed during co-culture with target and non-target cells in a dose-dependent manner. CAR-T cell activation was determined by release of IFN-γ into the supernatant and target cell depletion by flow cytometry. Both, T cell activation and cytotoxicity correlated with the amount of present target-antigen, while CAR-T cells did not react against target-negative cells. Furthermore, were CD4-positive HuT78 target cells also depleted, when present as a rare population in a mix with CD4-negative Raji cells.

Next, anti-CD4-DARPin CAR-T cells were tested against an HIV-latency model. Co-culture with HIV^+ J-Lat cells resulted in CD4-specific T cell activation and depletion of J-Lat cells pre- and post-reactivation with LRAs. Moreover, J-Lat reactivation led to a strong decrease of HIV expression after treatment with anti-CD4-DARPin CAR-T cells.

To examine the properties of anti-CD4-DARPin CAR-T cells in an autologous system, specific depletion was assessed by monitoring the $CD4^+$ and $CD8^+$ proportions of human T cells of the same donor over eight days. Upon CAR-expression, the $CD4^+$ population was clearly decreased, compared to the anti-CD30scFv-CAR control, while the $CD8^+$ T cell population persisted.

When the same experiment of autologous depletion was repeated in primary non-human primate T cells, the results were less obvious. Moderate reduction of the $CD4^+$ population was observed using rhesus CAR-T cells. In contrast, no CAR-mediated CD4-specific depletion was determined for primary T cells of AGMs.

4 Discussion

Anti-retroviral therapy has improved in the past years and patients' life fulfilment under best treatment is the same as that of a healthy person. Nevertheless, the use of CAR-T cells for the treatment for HIV infection is a reasonable approach based on the observation that CAR-T cells can control targeted hematopoietic cells in the long-term resulting in cure from leukaemia in a substantial number of cases (Wagner 2016). After derogation of anti-retroviral treatment, the risk increases that HIV positive cells switch from a latent into an activated state and produce infectious viral particles. Eliminating latently infected cells is thereby an urgent need to achieve cure from HIV infection (Speil 2009). Until now, no approach was able to overcome latency (Pillai and Deeks 2017).

4.1 Generation and functional testing of anti-CD4-DARPin CAR-T cells

Within the first part of this work (Section 3.1.1), it is described how the different CAR transfer vector plasmids (Figure 14) were cloned. Insertion of restriction sites of single cutting enzymes were inserted to enable the exchange of individual CAR domains. It was confirmed, that insertion of these restriction sites did not impact expression or functionality in comparison to the original CAR plasmid #1138 pBullet (Hombach *et al.* 1998) (data not shown). For detection of the CAR on the T cell surface, a directly labelled anti-human IgG antibody was used, which bound to the IgG spacer domain of the CAR. Because this IgG antibody is polyclonal, a tag should be inserted to use monoclonal antibodies. The intention was to achieve cleaner CAR^+ T cell populations in flow cytometry and assess the quantitative CAR expression levels, rather than just the amount of CAR^+ cells. Different tags, such as HA, His and Myc were inserted upstream and downstream of the CAR spacer domain. However, none of these tags could reflect the IgG staining (data not shown). One possible explanation could be the folded 3D structure of the CARs, by which the binding epitopes of the relatively small tags of only 6-10 amino acids are masked. CAR detection therefore remained using the polyclonal IgG antibody. After CAR transfer vector plasmids had been successfully cloned, MLV-based vector particles were generated. Detection of the particles by MLV-gag protein p30 is shown in Figure 17 and Figure 31. For all human- and nhp-based CARs, one version was generated carrying the wild-type (wt) CD28Lck domain and the equivalent version carrying the CD28DLck domain. For functional assays, only the CD28DLck versions were used. In parallel to the CAR vector component, PBMCs were isolated from buffy coats or fresh blood. Activation with T cell specific CD3 and CD28 antibodies resulted in very high T cell purity after 3 days (Figure 18). The same antibody clones were tested for specific activation of T cells of non-human primates (data not shown). However, staining of activation marker CD25 showed that anti-CD3 antibody clone

OKT-3 only activated human but not nhp T cells. Only stimulation with anti-CD3 clone SP34.2 resulted in upregulation of CD25 of nhp T cells and the typical clumping phenotype (Figure 19A, Figure 32). This finding indicates the importance of species-specificity of T cell activation domains. Although CD3 and CD28 have very high homology between humans and nhps, (Figure 30), the fact that they cannot be activated with the same antibody clones gives evidence that adjustment of the CAR activation domains to the T cell host is required for optimal T cell activation. Full assessment of the effect of species specificity of CAR activation domains towards T cell activation requires a cross-comparison of all T types with all CARs. However, this assessment was not focus of this thesis. Moving on to transduction with MLV vector particles, a protocol for purified and isolated T cells of human and nhps was established. For this purpose, T cells were transduced with eGFP transfer vectors, using various concentrations of vector particles (Figure 20). Expression of eGFP after 24h and 48h resulted in the transduction protocol described in Section 2.2.6. After conformation of transduction and CAR surface expression (Figure 21,Figure 33) in human and nhp T cells, functionality was evaluated in different assays. Co-culture with heterologous target and non-target cell lines allowed a defined and dose-dependent assessment of specificity, activation and cytotoxicity. To exclude background within the different readouts caused by T cells expressing the CD4 target antigen, they were depleted after PBMC activation. Figure 22 shows human PBMCs after magnetic separation of $CD4^+$ cells. The smaller and less granular T cells can be found on the bottom of both plots, at low SSC values. Besides these large T cell populations, another cell population was detected along higher SSC values but lower CD4 expression, compared to T cells. This population shows the typical pattern of monocytes in a flow cytometry dot plot. Monocytes are bigger and more granular than T cells and express lower levels of CD4. This decreased antigen expression is likely to be one of the reasons why the monocyte population cannot be fully depleted from the PBMC population and they remain to a small amount also in the CD4-negative fraction. However, following activation with T cell specific antibodies, remaining monocytes are cleared off, which is shown by staining of the T cell marker CD3. Figure 18 shows that after three days of activation, almost exclusively $CD3^+$ T cells are present.

For evaluation of functionality and specificity of human anti-CD4-DARPin CAR-T cells, they were at first co-cultured with antigen-positive target and antigen-negative non-target cell lines. In this setup, T cells expressing anti-CD30scFv CARs served as a positive control, since it is an established CAR (Hombach *et al.* 1998). Because HuT78 target cells were positive and Raji non-target cells were negative for both, CD4 and CD30 antigens (Supplementary Figure 2), they were suitable for both CARs in the same manner. A CAR carrying a DARPin which does not bind to human T cells, named H2C3, was used as a negative control, to show any unspecific background caused by the CAR framework. Figure 23 shows activation and cytotoxicity of human anti-CD4-DARPin CAR-T cells after 48h co-culture with heterologous target and non-

target cell lines. Figure 23A shows a clear dose-dependent secretion of IFN-γ, which increases alongside with the dose of CAR-T cells. This observation is also reflected by the cytotoxicity in Figure 23C. Anti-CD4-DARPin CAR-T cells induced IFN-γ release within the same range as anti-CD30scFv CARs. In contrast, co-culture with Raji non-target cells induced only minimal IFN-γ secretion (Figure 23). However, all samples which were expected not to induce T cell activation, all Raji samples and HuT78 samples with mock or DARPin H2C3, still showed some IFN-γ background. This background also increased together with the T cell dose. This observation can be explained with the fact that each co-culture still contained viable T cells. Although these T cells were transduced with a nonspecific CAR or mock control, they still retained the native T cell function. Upon contact with a "foreign" cell line, these T cell activating mechanisms were probably induced to a certain degree. For example, can primary T cells still recognise other antigens on the MHC of the cell line or react to cytokines it secreted. However, this IFN-γ background is up to 60-fold lower than in the target-specific sample. This conclusion was also reflected in the cytotoxicity (Figure 23C, D). Anti-CD4-DARPin CAR-T cells achieved the same or slightly higher cytotoxicity as the anti-CD30scFv control. At the same time, the non-binding DARPin-H2C3-CAR appeared to be non-cytotoxic towards the target cells. This observation is in line with the previous conclusion of some low unspecific T cell activation which at the same time does not induce killing. The same holds true for cytotoxicity towards Raji non-target cells (Figure 23D). Here, only a small amount of background was observed, which could be caused by the large number of cells within each individual well at E:T ratios from 1:16 to 1:2. Additionally, a dose-dependence of unspecific cytotoxicity towards Raji non-target cells was not observed. To further challenge the efficiency of anti-CD4-DARPin CAR-T cells, they were co-cultured with a mixture of target and non-target cells. In this setup, the proportion of HuT78 target cells was reduced by 50% within each step. Figure 24 shows that anti-CD4-DARPin CAR-T cells depleted even rare populations of HuT78 target cells very efficiently. Even at the lowest target cell fraction of 0.78%, anti-CD4-DARPin CAR-T cells were still capable of finding and depleting around 40% of target cells within 48h. However, the unspecific depletion of Raji non-target cells was in this case higher than with a pure target or non-target population. One potential reason could be the release of IFN-γ by activated CAR-T cells. Cytokine release can induce activation of surrounding T cells, including untransduced ones. This activation can result in increased T cell proliferation and killing due to antigen recognition by the native TCR. Efficient elimination of a rare target population is a significant result because, in contrast to patients with hematologic malignancies, the number of target cells decreases to less than 100 $CD4^+$ T cells per µl blood as AIDS progresses (Hoffmann and Kamps 2007) while targeting leukemic cells is facing an overwhelming number of target cells. It is therefore essential, that CAR-T cells deplete the cellular HIV reservoir although the target population is extremely rare, either because of viremia or towards the end of a CAR-T cell treatment, when the majority of

target cells has already been eliminated. On the other hand, every single HIV$^+$ target cell which remains in the patient bears the risk of a reviving viral spread. Anti-CD4-DARPin CAR-T cells have been shown to deplete the CD4$^+$ target population specifically and efficiently down to a very rare target population.

The efficacy of anti-CD4-DARPin CAR-T cells was also tested in a model of HIV$^+$ target cells (Section 3.3). Overall, anti-CD4-DARPin CAR-T cells were activated and depleted J-Lat target cells in the same dose dependent manner as HIV negative HuT78 cells (Figure 23; Figure 26). However, the total amount of secreted IFN-γ was much lower compared to HuT78 co-culture. This decreased IFN-γ release could be caused by the lower expression levels of the CD4 target antigen (Supplementary Figure 2). If a CAR-T cell has less target receptors to engage with, less CARs are activated to trigger downstream T cell activation pathways which lead to IFN-γ secretion. Nevertheless, the depletion of HIV$^+$ J-Lat target cells was very efficient (Figure 26), despite the low levels of antigen expression. This is of biological relevance since CD4 is often down-regulated upon HIV infection (Bresnahan *et al.* 1998). Complete elimination of the viral reservoir is required (Finzi *et al.* 1999) in order to prevent reactivation of latently infected cells and de novo viral spread. This was shown in Figure 26 and Figure 27. By depleting the cellular CD4$^+$ reservoir, further expression of HIV and therefore forming of new viral particles could be prevented. In addition to the latent cellular reservoir, CD4-DARPin CAR-T cells were also capable of depleting activated CD4$^+$ T cells (Figure 28). For the depletion of J-Lat cells, lower E:T ratios were chosen, because of the generally lower viability of this transgenic cell line, compared to HuT78. High E:T ratios are associated with higher total T cell numbers per well which negatively impacts the target cell viability. One of the major limitations of the J-Lat latency model is the secretion of incomplete viral particles. Because of the depletion of HIV env, the produced viral particles are not able to re-infect new cells. Investigation of the dynamics of the cellular HIV reservoir requires a model of latently infected primary T cells, producing fully replication competent HIV particles upon activation. This laboratory setup is extremely difficult (Jacobson and Khalili 2018). To infect them *in vitro* with HIV, primary T cells need to be brought into a highly activated state, similar to CD3/CD28 activation in preparation of transduction with retroviral particles. As mentioned previously (Section 1.1.5), the exact mechanisms inducing the latent state of an infected cell remain unknown and can therefore not be induced very well in a laboratory assay. The next key step for the validation of the approach would be testing of anti-CD4-DARPin CAR-T cells in an immunocompetent animal model. In this case, the only suitable model is the treatment of SIV$^+$ non-human primates with autologous anti-CD4-DARPin CAR-T cells. This *in vivo* experiment should address multiple questions: First, do anti-CD4-DARPin CAR-T cells show sufficient efficiency to eliminate the entire viral reservoir or are there any organs or tissues bearing HIV$^+$ cells which CAR-T cells don't access? Second, can the animal survive during full depletion of CD4 T cells and will CD4 T cells revive after termination

of the therapy? Further questions tackle the effects of CAR-T cell cytokine release on the latent reservoir. Because only $CD8^+$ but not $CD4^+$ CAR-T cells can be used to deplete a CD4 target, a long-term immunocompetent animal study could potentially give an indication, whether the shorter persistence of CD8 CAR-T cells compared to CD4 CAR-T cells is sufficient to deplete the entire viral reservoir. Additionally, one of the most important questions is the possibility of off-target effects so that CAR-T cells cause damage to any other tissue than $CD4^+$ T cells. Although a functional nhp study could not be performed during this thesis, preparatory *in vitro* assays were carried out. One important step was to test functionality and efficacy of anti-CD4-DARPin CAR-T cells in an autologous setting. Therefore, human primary T cells were transduced to express anti-CD4-DARPin CAR and the presence of $CD4^+$ T cells was monitored over time. Figure 29 shows the decrease of the $CD4^+$ T cell population, while $CD8^+$ T cells remain. This setting also shows clearly why CD30, although a potential target for CAR-T cell therapy of cancer, is not suitable against HIV: Compared to cell lines, primary T cells, if activated or not, do not express sufficient amounts of CD30 to be efficiently depleted by CAR-T cells. In this case, anti-CD30scFv CAR only served as a control CAR for the *in vitro* assays. The slight increase of the $CD8^+$ T cell population in mock and CD30scFv could have been caused by the T cell activation in preparation of transduction with retroviral particles. For heterologous killing of immortalised cell lines, the $CD4^+$ population was depleted prior to T cell activation and transduction. Therefore, transduced cells could be activated and expanded without any CAR-T mediated killing. This activation was then terminated by reduction of IL-2 to a minimum level. For autologous killing assays, this shut down of T cell activation was not possible because anti-CD4-DARPin CAR-T cells were constantly exposed to the CD4 target so that the depletion of CD4 T cells started shortly after transduction. Therefore, activated T cells are likely to express low amounts of CD30 which could have induced some amount of depletion. Nevertheless, specific and efficient depletion of autologous human $CD4^+$ T cells by anti-CD4-DARPin CAR-T cells could be shown. In preparation for a nhp study, the same experiment was repeated with primary T cells of rhesus macaques and *African Green Monkeys* (AGMs). To achieve full T cell activation, the intracellular CAR activation domains were adjusted to the CD3 and CD28 T cell receptor domains of the respective host species (Figure 30). The fact that the activation of nhp T cells required the use of a different CD3 antibody clone than for human T cells already indicates the potential need of a species-specific CD3zeta chain to achieve sufficient CAR-mediated T cell activation. The results for autologous depletion of nhp $CD4^+$ T cells were not as clear as for human T cells (Figure 34). However, the results give indication that at least the rhesus model is suitable. In T cells of both tested rhesus macaques, a reduction of the $CD4^+$ T cell population could be observed in presence of anti-CD4-DARPin CAR. However, this reduction was less than for human T cells, eight days post transduction and the difference between the anti-CD4-DARPin and the anti-CD30scFv samples

were less clear. This result could have different reasons. The cross-reactivity of anti-CD4-DARPin_57.2 was shown previously (Schweizer *et al.* 2008). However, CD4 proteins of *homo sapiens* and *macaca mulatta* are only 92% homologous in peptide sequence (Uniprot). It is therefore possible that the binding affinity of DARPin_57.2 is lower towards CD4 of rhesus than towards human CD4. A potentially lower expression of CD4 on the T cell surface of rhesus macaques is less likely to have an impact on efficacy of anti-CD4-DARPin CAR-T cells, since the killing of low CD4-expressing J-Lat cells was highly efficient. Another reason for an impaired depletion of rhesus $CD4^+$ T cells could be the low transduction efficiency and resulting low CAR expression (Figure 33 A, B). Activated T cells were transduced with MoLV based gamma-retroviral particles carrying a Gibbon ape Leukaemia Virus envelope protein for cell entry. The rationale for choosing MLV as CAR gene transfer vector rather than a lentiviral vector, which is widely used in the clinics, can be found in the host-pathogen interaction. Lentiviral vectors are based on HIV. After cell entry, cellular factors are involved in uncoating of the particles and reverse transcription of the viral RNA. It was shown that cellular restriction factors, such as tripartite motif-containing protein 5α (TRIM5α), can prevent uncoating of the virus and thereby inhibit permanent integration of viral provirus into the host genome (Stremlau *et al.* 2006; Sebastian and Luban 2005). Whether inhibition of viral integration is successful or not depends on the combination of virus and host. TRIM5α of old world monkeys, such as African Green Monkeys, showed to prevent uncoating of HIV and thereby also lentiviral particles (Kratovac *et al.* 2008; Stoye 2002). Well established MLV-based gene transfer was therefore the best choice for all three T cell donor species. Although transduction and CAR expression was confirmed, only around 10% rhesus T cells were CAR positive on day 3 post transduction. The lower number of CAR-T cells within the population could have led to impaired CD4 T cell depletion by anti-CD4-DARPin CAR-T cells in rhesus. Further evaluation of other gene transfer-vehicles, such as SIV-based vector particles might lead to an increased transduction efficiency and CAR expression. T cells of AGMs on the other hand, did not show any reduction of the $CD4^+$ T cell population at all. At the same time, a trend regarding $CD4^+$ and $CD8^+$ populations was observed between mock, aCD30scFv and aCD4-DARPin CAR samples. The cause of these results in AGM T cells could be similar to those described for rhesus macaques. Cross-reactivity of DARPin_57.2 with AGM CD4 was not tested. The same lack of cross-reactivity could also be a major issue for fluorescently labelled antibodies. These antibodies were used to detect the different T cell populations by flow cytometry. However, the majority of commercially available antibodies is derived to bind human proteins. Depending on the sequence homology and the binding epitope, antibodies are fully, partially or not cross-reactive between species. Flow cytometry plots in Supplementary Figure 6 and Supplementary Figure 7 indicate an impaired binding affinity of the used CD4 and CD8 antibodies, since the

two populations could not be fully separated. However, several CD4 and CD8 specific antibodies had been tested but the ones used for these experiments showed to be the most suitable options (data not shown). Because HIV emerged as an adaption of SIV to humans as a new host (Wertheim and Worobey 2009), SIV in nhps is the most suitable animal model to study HIV life cycle, viremia, immunology and disease progression (Chen 2018). Most important for the approach of targeting CD4 is that SIV and HIV both target the same cell populations in the host and use CD4 as a cell entry receptor. Generation of nhp-based anti-CD4-DARPin CAR-T cells is therefore a logical step.

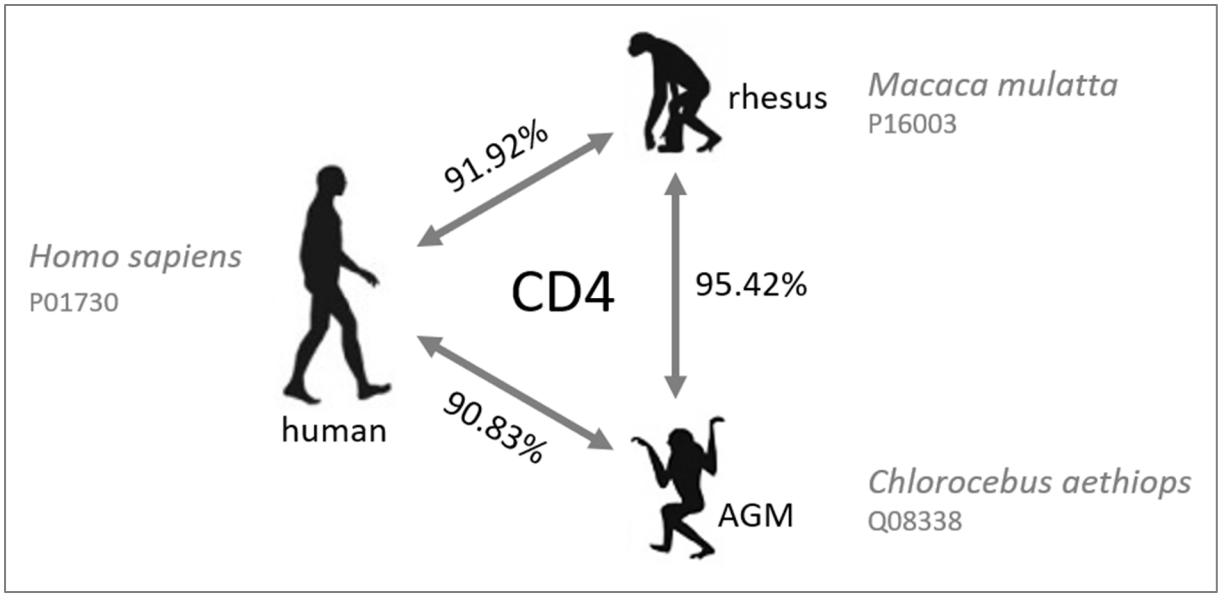

Figure 35: Species homology of CD4.

Homology of the peptide sequences of the CD4 precursor protein between human, rhesus macaques and African Green Monkeys. Source: Uniprot

The approach described in this thesis takes advantage of the specificity and efficiency of CAR redirected T cells in eliminating autologous target cells. It has been demonstrated that CAR-T cells, directed against CD4, bind, and deplete $CD4^+$ T cells in a very specific and efficient manner. Binding of CD4 leads to activation of the CAR-T cell and specific killing of the target cells, whereas no reaction is triggered by cells lacking CD4. Furthermore, $CD4^+$ cells were efficiently depleted even at small numbers within a heterogeneous population of non-target cells. Depletion occurred to equal efficiency regardless of high or low CD4 expression levels. Upon incubation with an HIV^+ T cell line, DARPin CAR-T cells efficiently depleted the target in a pre and post activation setup. Besides heterologous cell lines, anti-CD4-DARPin CAR-T cells were also capable of specific depletion of autologous primary $CD4^+$ cells. In order to further investigate anti-CD4-DARPin CAR-T cells in an immunocompetent animal model, non-human primate-based CARs were generated, containing rhesus and AGM activation domains. Both CARs could be successfully expressed on the T cell surface of the respective species. In an autologous setting, rhesus DARPin-CAR-T cells depleted $CD4^+$ T cells specifically.

4.2 CD4 as a target for CAR-T cell therapy against HIV

Approaches for treatment of HIV are following two main strategies. Most of them are aiming for a functional cure, meaning a defined long-term control preventing disease symptoms and viral spread. However, in this case the pro-viral reservoir is not fully eliminated but supressed. In a functional cure, the therapeutic agent will always have to be active or ready to be activated, in any event viral replication. A functional cure therefore bears some risks and drawbacks. On the one hand, the currently used cART, by which a combination of drugs inhibits multiple steps within the viral replication cycle. cART requires continuous life-long medication of the patient, cost-intensive and with more or less severe side-effects as discussed in Section 1.1.4. Cell therapies have been under investigation to cure HIV infection for a few decades (Wagner 2018), aiming to prevent viral spread without the need of ART. Multiple approaches are aiming for a functional cure of HIV, which have to overcome various challenges. Extremely low antigen expression levels impede the chance to capture the virus. Increase of efficiency requires targeting of highly conserved or non-escaped viral epitopes. This can be accomplished by broadly neutralising antibodies (bNAbs). bNAbs were shown to bind highly conserved regions, mainly in gp120 of HIV env protein (Bournazos *et al.* 2016; Huang *et al.* 2016). Because the binding epitopes are presented on a broad variety of different mutants, bNAbs are capable of neutralising a broad variety of HIV strains (Xu *et al.* 2018). But to be efficacious against reoccurring viral replication, also a sufficient number of effector T cells would have to be recruited to deplete virus producing cells. Therefore, also CAR-T cells derived from bNAbs have shown to specifically eliminate HIV infected cells (Ali *et al.* 2016; Ghanem *et al.* 2018). In addition to

efficacy, anti-HIVenv CAR-T cells could also be engineered to be resistant against HIV infection (Hale *et al.* 2017). However, in order to control viral replication, anti-HIV CAR-T cells will have to be persistent and functional over the entire lifespan. However, if cytotoxic T cells have been strongly activated before levels of target antigen decrease rapidly, they tend to stop proliferating, show exhaustion and enter apoptosis. Life-long persistence and potency of HIV-specific CAR-T cells in an infected patient are essential, as well as a good penetration of tissues to deplete hidden viral sanctuaries. As a result of improved CAR design, using second, third or fourth generation signalling domains (Figure 6) and advanced binding domains, efficacy of CAR-T cells against HIV infected cells has improved (Leibman *et al.* 2017). Whether improved CAR-T cells can sustain permanent virological control after cessation of cART remains subject to long-term studies.

The hurdles of a functional cure are very low to inexistent antigen expression, viral heterogeneity due to mutational escape and T cell dysfunction or exhaustion. But the major caveat which prevents a sterilizing cure, meaning the full elimination of the complete viral reservoir, is the inaccessibility of the latent reservoir cells. bNAbs and anti-HIV CAR-T cells, targeting viral proteins can only deplete cells which are actively replicating and translating those. E. g. HIV gp120 antigen is only expressed by infected cells upon reactivation. In this context, CAR T cells targeting gp120 can deplete ACH-2 cells which was used as a model for latently infected cells(Sahu *et al.* 2013; Wagner 2016; Hale *et al.* 2017). However, the ACH-2 cell line constitutively produces low levels of HIV proteins, reverse transcriptase and p24. On the contrary, latently infected blood cells do not express HIV gp120 (Pillai and Deeks 2017) which would therefore not be recognized by the respective CAR-T cells.

The latent reservoir cannot be targeted through viral proteins. Extensive research and development did not reveal a cellular marker to identify cells with integrated viral DNA sequences (Richman 2017).One of the most recent candidates was CD32a. After depletion of the $CD32a^+$ population within latently infected cells, the expression of HIV following T cell reactivation was strongly decreased(Descours *et al.* 2017). Nevertheless, CD32a was not expressed by all latently infected cells leaving some CD32a-negative cells behind which may gave rise to virus re-activation (Badia *et al.* 2018). Depletion of $CD32a^+$ cells is therefore expected to induce rather a delay in viral replication than eradication of the entire pool of infected cells (Pillai and Deeks 2017; Richman 2017). The very low possibility for the existence of a cellular latency marker is described in 1.1.5 and 1.2.2. In a state in which only very low amounts of viral RNA are transcribed, it is unlikely that other cell pathways are influenced by the presence of the integrate. The last key factor in the HIV life cycle before entering the latent state, is the cell entry receptor CD4 (Chan and Kim 1998). This means that not every $CD4^+$ cell is positive for HIV but every cell which is positive for HIV does express CD4. Consequently, depletion of

$CD4^+$ cells would completely eliminate the viral reservoir, including latently infected cells. Cytolytic T cells engineered with a chimeric antigen receptor (CAR) which recognizes CD4 and redirects T cells towards $CD4^+$ cells, aim to deplete defined cells from the blood stream in a very specific and efficient way. In this context, CD4 is a valuable target for redirected cell therapy because CD4 is the exclusive cell entry-receptor for HIV (Wilen *et al.* 2012).

To date, only one patient is suspected to have achieved a sterilising cure, in which HIV is completely eliminated from the body. The so-called “Berlin patient” had received cART to control its HIV infection for over ten years when he was diagnosed with acute myeloid leukaemia (AML) along with a dramatic drop in CD4 T cell count (Speil 2009). To treat AML, the patient received a $CD34^+$ stem cell transplant of an HLA-identical donor which was homozygous for the delta32 mutation of CCR5, one of the co-receptors for HIV cell entry. If the delta32 deletion occurs on both alleles, entry of HIV particles into the target cells is inhibited (Philpott *et al.* 1999). Vertical spread of HIV strains with CCR5 tropism is therefore suppressed (Sorà 2002) and patients appear to be largely immune against these viruses. After the Berlin patient received the Δ32/Δ32 stem cell transplant, cART has been discontinued until present, without any signs of viral spread or detectable viral RNA alongside with stable CD4 T cell counts (Speil 2009; Jessen *et al.* 2014; Allers *et al.* 2011). The approach of depleting the vast majority of T cells, which also carries the latent reservoir, which is then replaced by a stem cell transplantation has been undertaken in the two “Boston patients”. Both patients were heterozygous for Δ32_CCR5 but the respective donors did not carry the mutation, which made the cells after maturation susceptible for HIV. Without additional cART, replication-competent virus was produced and viral rebound observed after 3-7 months, in both cases (Henrich *et al.* 2014). The Boston patients set a very concrete example of the crucial role of the viral reservoir. Although preconditioning depleted viral host cells to a minimum, enough provirus obviously survived to generate compete and infectious viral particles. Prevention of infection of T cells derived from the stem cell transplant with HIV, requires an HLA-matching donor, homozygous for the Δ32_CCR5 deletion. Because of the limited number of volunteers for stem cell donations, of which only 1% are happen to carry the Δ32/Δ32 mutation (Laurichesse *et al.* 2007), an expansion of this method across the majority of HIV patients does not appear feasible. An example of how quickly a viral reservoir can be established, sufficient to cause a chronic infection is depicted by the case of the “Mississippi baby”. A new-born child, prenatally infected with HIV, received cART 30 hours to 18 months after birth to minimise the establishment of a viral reservoir. The child then remained without any detectable virus for 2.4 months post termination of cART, before it relapsed into viral rebound (Luzuriaga *et al.* 2015). Both cases, the “Boston patients” and the “Mississippi baby” highlight the exiguous window in which the latent HIV reservoir can be affected: The time to prevent establishment of the viral reservoir, as well as the

size required to enable viral rebound. Only a vaccine, effective against 100% of all HIV strains could prevent infection after HIV exposure. However, because of the high viral mutation rate, such a vaccine remains undiscovered (Haddox *et al.* 2016). It is therefore inevitable to find a method which is capable of depleting the entire viral reservoir from the patient, without any residues, competent to allow viral rebound. The results presented in this thesis have shown that the specific depletion of the $CD4^+$ cellular HIV reservoir by anti-CD4-DARPin CAR-T cells has the potential to fulfil these qualifications. The Berlin patient is to date the only known individual, remaining devoid of detectable levels of HIV without any antiretroviral treatment for over a decade (Jessen *et al.* 2014). Admittedly, the final proof whether a sterilising, hence the complete eradication of the viral reservoir, or a functional cure has been achieved, is still outstanding. This would require the investigation of every single potential host cell within the patient. Cromer *et al.* 2017 have yet performed statistical computations, reasoning that an average of 20-year treatment-free remission from HIV equates to a reduction of the frequency of viral reactivation by 99.9%. This means that, should the Berlin patient still harbour a replication-competent viral reservoir, its reactivation has been reduced to less than 0.1%, since he stopped cART in 2007 (Speil 2009). This observation supports the presumption, that it is possible to effectively deplete the viral reservoir. Anti-CD4 DARPin CAR-T cells likely fulfil the criteria: J-Lat cells which are latently infected cells were eradicated, shown by the decreased expression of HIV-GFP (Figure 26). By elimination of the latent cell reservoir, the probabilities for reactivation and production of new virus were minimized. Specific depletion was not only shown for the J-Lat cell line but also for autologous $CD4^+$ T cells mimicking the cellular situation during CAR therapy.

4.3 Risks and benefits of targeting CD4

The two most important questions when targeting and depleting $CD4^+$ cells are whether and how a patient can survive the depletion of its entire $CD4^+$ cell population and if the mature CD4 T cell population can sufficiently recover from naïve stem cells after successful treatment. Depletion of the entire CD4 T cell population puts a patient in nearly the same place as during the acute late phase of AIDS, a rapid drop in CD4 T cell count (Figure 1) - undoubtedly, a very critical state. These patients would be severely immunocompromised with very little defence mechanisms against other pathogens. Nevertheless, under intensive medical care, immunodeficiency can be managed. One example, although less severe, is the depletion of $CD19^+$ B cells during approved CAR-T cell therapies (Mullard 2017). Patients are lacking B cells and are therefore the ability to produce neutralising or depleting antibodies. This state must be sustained until the risk of disease relapse has been obviated. A graver situation arises during depletion of T cells. The complete absence of T cells is comparable to that of SCID (severe

combined immunodeficiency). SCID patients carry a genetic disorder, resulting in dysfunction of B cells, T cells or both (Fischer 2000). Due to the lacking adaptive immune response, SCID patients require intensive medical care and a sterile environment. Although the mortality rate in early infant years is very high, some patients survived over 10 years. Translated to specific depletion of $CD4^+$ T cells for the treatment of HIV, the situation is expected to be less severe. Compared to SCID, only the T helper cell population is depleted, whereas innate immunity remains, as well as B cells, dendritic cells and $CD8^+$ effector T cells. Therefore, a predominant part of the immune system would remain functional during anti-CD4 CAR-T cell treatment. Overall, there is a realistic chance that depletion of CD4 cells would be tolerated by the patient, under appropriate medical care. An immunocompetent animal model, such as SIV infected nhps, could provide information about the most severe issues and possible countermeasures, e.g. passive immunisation or application of cytokines to maintain immune activation. Depletion of $CD4^+$ T cells with monoclonal antibodies in non-human primates have already shown to be safe and specific(Jonker *et al.* 1993).The second question around targeting $CD4^+$ cells would also have to be addressed in an immunocompetent animal model: the recovery of $CD4^+$ T cells after termination of CAR-T cell therapy. Again, comparing to the clinical application of CD19 CAR-T cells against B cell malignancies, some technologies might also be advisable after CD4 T cell depletion. In order to recover the B cell population after CAR-T cell therapy, multiple options are currently under investigation. The simplest but also rapid one is administration of cortisone. Cortisone is an immunosuppressive steroid hormone, which reduces inflammation as during autoimmune reactions or transplant rejection. However, cortisone does not specifically deplete CAR-T cells, but rather suppresses activation of the majority of immune cells. A more promising approach is the development of inducible CARs or switch technologies (Hoyos *et al.* 2010; Ciceri *et al.* 2009). Straathof *et al.* 2005 developed a suicide switch system in which a modified human caspase 9 fused to a human FK506 binding protein (FKBP) is co-expressed with the CAR. Application of a small molecule allows conditional dimerization followed by induction of apoptotic intracellular pathways. Another way of specific depletion of CAR-T cells is mediated by monoclonal antibodies. Co-expression of a truncated version of epidermal growth factor (EGFRt) has been used as a binding domain for IgG1 monoclonal antibody cetuximab (Paszkiewicz *et al.* 2016). After specific depletion of $CD19^+$ target cells in sub lethally irradiated WT C57BL/6 mice, animals were injected with two doses of cetuximab, which resulted in complete elimination of CAR-T cells and permanent reversion of B cell aplasia in these mice. The same or similar switch technologies could be applied for anti-CD4 CAR-T cells to provide a more controlled CAR-T cell activity and enable recovery of the $CD4^+$ cell population. Recovery of the $CD4^+$ T cell pool after withdrawal from therapy will require intensive investigation also because human T cells are derived and matured in the thymus mainly during childhood. When entering adulthood, activation of the thymus is more and more reduced. It was shown that the

$CD4^+$ T cell pool in patients after chemotherapy recovered only conditionally due to insufficient thymic activity (Hakim *et al.* 1997). However, upon application of CD4-specific depleting antibodies to chimpanzees, the $CD4^+$ cell population was decreased below 5% of total blood cells (Jonker *et al.* 1993). Despite multiple dosing, no serious infections were observed. The population of $CD4^+$ T cells re-established within three weeks after withdrawal from therapy (Policicchio *et al.* 2016; Jonker *et al.* 1993). Furthermore, is the depletion of immune cells a common tool used in cancer therapy. Chemotherapy is the most widely used treatment for cancer patients, whereby cells with a high proliferation rate, such as cancer but also healthy cells are depleted. Depending on the type of chemotherapy, immune cell counts can be severely decreased (Steingrimsdottir *et al.* 2000). Monitoring of leukocyte counts of leukaemia and lymphoma patients undergoing chemotherapy revealed a rapid drop of $CD4^+$ T cells below 10 cells/µl (Fagnoni *et al.* 2002; Mackall *et al.* 1995). Reconstitution of $CD4^+$ T cells was observed in the majority of patients, however, this was highly dependent on age and thymopoiesis (Mackall *et al.* 1995). During preconditioning, lymphocytes are irradiated prior to adoptive T cell transfer, aiming to minimize the number of regulatory T cells. These would decrease the efficiency of the tumour-reactive cytotoxic T cells(Wrzesinski *et al.* 2010; Muranski *et al.* 2006). Subsequent transplantation of stem cells lead to a good prognosis of patient survival(Dudley *et al.* 2005). Transplantation of $CD34^+$ stem cells could also be considered for patients failing to sufficiently recover their $CD4^+$ T cell population.

The risk of on-target/off-tissue toxicity by anti-CD4 CAR-T cells is considered very low, since CD4 is a unique target expressed mainly by T cells, monocytes and macrophages (The Human Protein Atlas 2018). Specificity of CAR-T cells towards their target antigen can be tested by co-culture with cell lines or tissues bearing potential risk of off-target or on-target/off-tissue toxicity. If CAR-T cells are not activated in presence of these tissues, unspecific toxicity is unlikely. The only potential risk regarding on-target/off-tissue toxicity is expected in neuronal cells of the cerebral cortex. Medium levels of CD4 protein expression was detected in immunohistochemistry stains with two out of four tested monoclonal antibodies (The Human Protein Atlas 2018). CD4 expression by neuronal cells is a known and serious risk for therapies targeting CD4 (Omri *et al.* 1994). Toxicity of anti-CD4 CAR-T cells against neuronal cells could cause serious if not life-threatening incidents. However, CD4 serves as HIV cell entry receptor also in neuronal cells, which makes them a potential viral reservoir (Peudenier *et al.* 1991). Depletion of these cells should not be considered, since damage of neuronal cells bears a high risk of irreparable damage. In this case, prompt administration of antiretroviral therapy could potentially prevent HIV infection of neuronal cells, since these are usually not the primary HIV target, rather than T cells or mucosal tissues. One way to prevent on-target/off-tissue toxicity against neuronal tissues could be bi-specific CARs (Ghanem *et al.* 2018). On the one hand,

tandem- or conditional CARs, triggered only by binding of both target antigens could limit cytotoxicity to a certain population of target cells, e. g. CD4/CD3 for T cells. Another option could be the combination of CD4 and a marker of the neuronal lineage in inhibitory CAR-T cells (Figure 37E). Hereby, CAR-T cells would be reactive against $CD4^+$ but not neuronal cells. These concepts of improving cytotoxicity of CAR-T cells are currently under investigation (Zhang and Xu 2017). For both cases, use of DARPins could be beneficial, since the small molecular size favours insertion of multiple binding domains. In the case of anti-CD4 CAR-T cells based on binding domain DARPin_57.2, it has yet to be demonstrated, whether this domain is also reactive to human neuronal cells. Because not all antibodies showed reactivity against neuronal tissues, there is evidence that also some DARPin clones might be less affine towards CD4 on neurons. This assumption is also reinforced by the observation of depleting CD4 antibodies, which did not cause any damage to neuronal tissues in chimpanzees (Jonker *et al.* 1993). An example for anti-CD4-DARPin CAR-T cells is shown in Figure 23 B and D. Anti-CD4-DARPin CAR-T cells were specifically activated and induced target depletion of $CD4^+$ HuT78 or J-Lat, but not $CD4^-$ Raji cells.

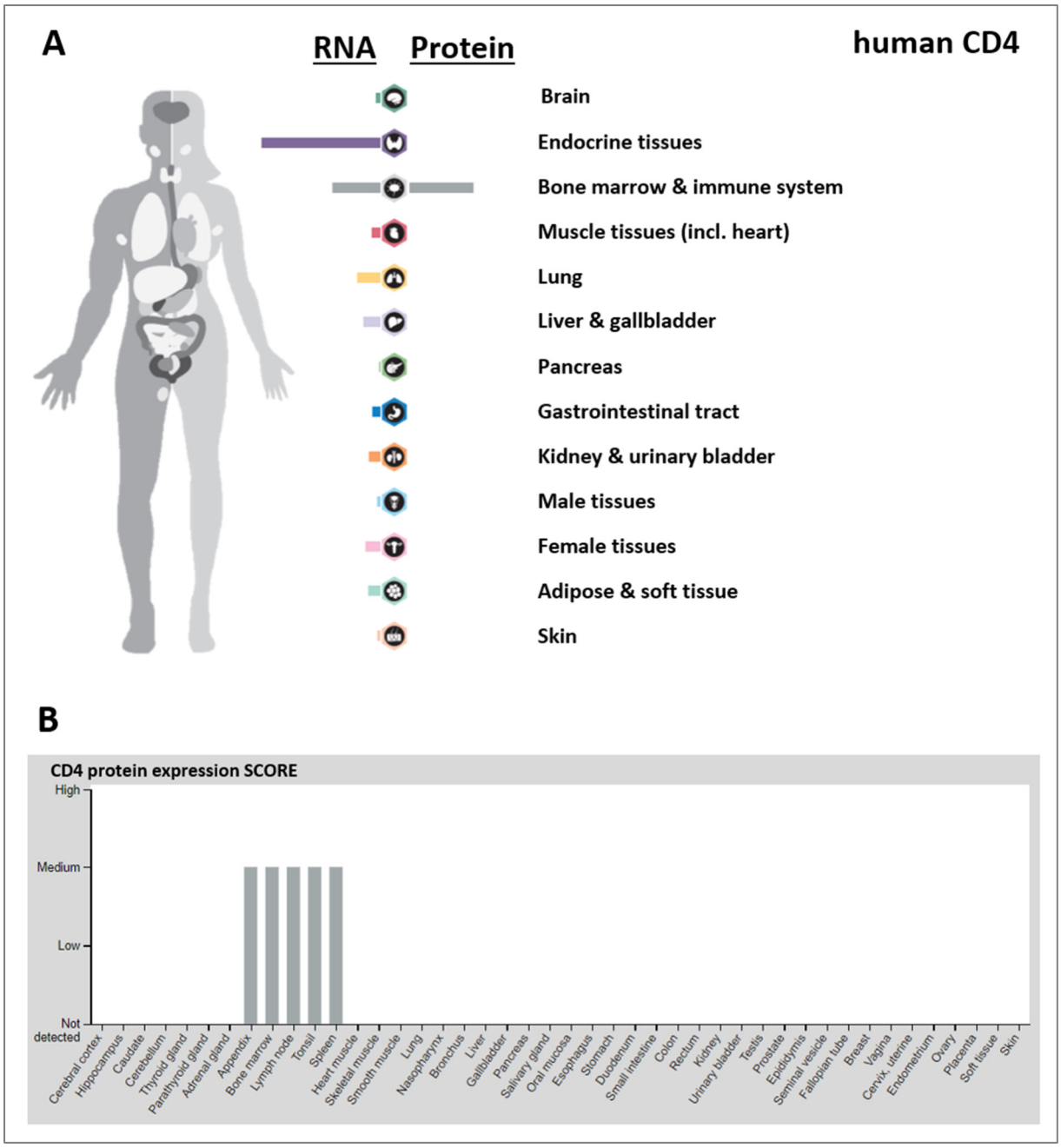

Figure 36: CD4 expression in human tissues.

Expression of human CD4 based on RNA and protein levels. **(A)** left: RNA levels of CD4 RNA-seq results generated in HPA are reported as number of transcripts per million (TPM). Each bar represents the highest expression score found in a particular group of tissues. Right: Protein expression scores are based on a best estimate of the "true" protein expression from a knowledge-based annotation. For genes where more than one antibody has been used, a collective score is set displaying the estimated true protein expression. **(B)** CD4 protein expression data for 44 human tissues. Color-coding is based on tissue groups, each consisting of tissues with functional features in common.
Source: The Human Protein Atlas 2018

The use of genetically modified cells in HIV or Oncology patients might also raise the concern of possibly occurring recombination events, resulting in the generation of new replication-competent viral particles. Although vector particles, such as MLV used in this project, are designed as replication incompetent, a theoretical chance remains, that integration of the therapeutic transgene into the host cell might be followed by genetic recombination. If this recombination resulted in the generation of novel replication-competent particles, safety and control of CAR-T cells and other gene therapy products would have to be questioned. However, long-term monitoring of numerous patient samples from various gene-modified T cell products showed no indication of replication-competent retrovirus/lentivirus or insertional oncogenesis, regardless of the transgene delivery systems used, retroviral, adenoviral or lentiviral vectors (Marcucci *et al.* 2018). Another option to prevent genesis of recombinant virus could be non-viral gene-transfer, such as sleeping beauty transposons (Hudecek *et al.* 2017) or non-viral RNA transfer (Svoboda *et al.* 2018).

4.4 Future perspective of CAR-T therapies against HIV

Previous attempts to use CAR-T cell therapy for the treatment of HIV haven been targeting viral proteins, mostly HIV env. CAR-T cell targeting to gp120 (Wagner 2016; Ali *et al.* 2016; Liu *et al.* 2015) harbours the conceptual weakness that the mutation rate of HIV proteins is extremely high (Cuevas *et al.* 2015) and targeting gp120 does not deplete latently infected cells. An alternative strategy is stimulating the latently infected cells to induce the expression of viral envelope proteins which then are targets for CAR-T cell therapy The so-called “Shock-and-Kill” or “Kick-and-Kill” approach uses LRAs, such as HDAC inhibitors, to initiate transcription and synthesis of viral proteins (Yang *et al.* 2018; Jones *et al.* 2016; Xu *et al.* 2017). Following expression of viral proteins, cells are then depleted by HIV-specific antibodies or cytotoxic immune cells. However, this strategy harbours the risk of viral spread during the induction phase. Moreover, secreted cytokines induce surrounding cells to become activated and to produce infectious particles. An additional challenge is again the heterogeneity of the viral reservoir. Not all latently infected cells are equally susceptible for reactivation. Depending on the site of integration, phenotype and anatomical location of the latently infected cell, one mechanism to reverse HIV latency could be insufficient to reactivate all subtypes of the latent reservoir. The major reservoir for latent HIV is resting memory T cells (T_M), in which downstream TCR pathways are depleted (Geisler 2004). Hence the question is raised, whether one method of latency reversal can be developed to induce expression of viral proteins in resting memory T cells. Life-long control over viral replication by anti-HIV-CAR-expressing or HIV affinity enhanced T cells requires long-term persistence and function. To maintain activity of HIV-targeted T cells, multiple infusions will be needed presumably. Size and heterogeneity of the

latent reservoir within every individual patient will drive efficiency of this approach (Richman 2017; Ho *et al.* 2013). In contrast, targeted elimination of, as the entire CD4 population could potentially eliminate the reservoir of diseased cells by one treatment analogous to CAR-T cell mediated elimination of the malignant cell pool of patients with leukaemia (Maude *et al.* 2014). In recent years, various designs of CARs with different activation mechanisms have been developed (reviewed by (Hartmann *et al.* 2017). Figure 37 gives a broad summary over recent CAR designs. Besides the prefiguration CAR (Figure 37A) with a specific binding domain, fused to second or third generation intracellular activation domains, bispecific CARs were developed (Figure 37 B-D). Expression of two CARs by the same T cell (multi CAR-T) enables redirection against two different targets of which both are capable of inducing T cell activation. Integration of two transgenes is likely to reduce T cell transduction efficacy, proliferation and persistence. Tandem CARs synergise bi-specificity within one CAR but require presence of both binding epitopes on the same target cell. In terms of HIV targeting, bispecific CARs could potentially enable targeting two different viral epitopes and thereby reduce the chance of escape mutations. Conditional CARs strictly require expression of both target antigens, while inhibitory CARs prevent T cell activation upon expression of a non-target antigen (Figure 37E). This method can be useful to prevent cytotoxicity towards non-target tissues. Intracellular or extracellular switch technologies have been developed to gain control over CAR-T cell activation Figure 37F, G). Hereby, small molecules are systemically applied to enable CAR activation. Extracellular switches consist of CARs, directed towards a bispecific molecule which also binds to the target epitope. Bridging of the CAR-binding domain to the target epitope then initiates T cell activation. Intracellular switches on the other hand link the intracellular CAR activation domain with the co-stimulation domain. Only in presence of the connecting intracellular molecule, sufficient T cell activation is obtained. Switch technologies are of particular interest as a potential combination with anti-CD4 CAR-T cells. To allow recovery of $CD4^+$ T cells after depletion of the cellular reservoir, withdrawal of the switch molecule could potentially inactivate anti-CD4 CAR-T cells. In addition, CAR-T cells, engineered to secrete proliferation-stimulating cytokines (Chmielewski and Abken 2015) or prevent suppression of T cell activation by checkpoint inhibitors, such as PD-1 could potentially have an increased activation and persistence in the patient, enhancing chances of efficient depletion of the viral reservoir. Alternatively, binding of the target antigen can be controlled by inducible CAR expression (Cartellieri *et al.* 2016) allowing a balance between depletion and subsequent recovery of $CD4^+$ cells.

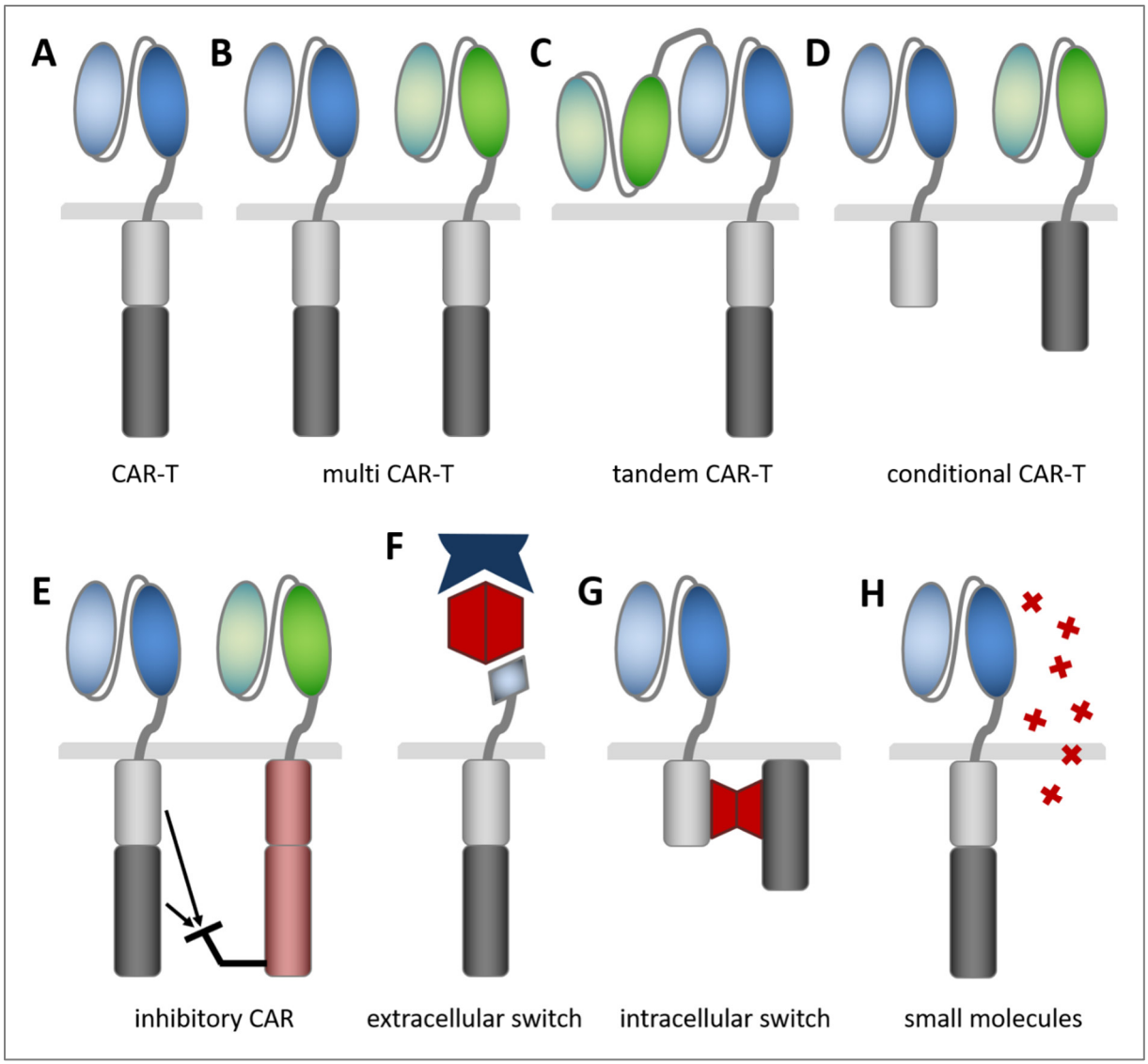

Figure 37: Overview of CAR technologies and designs.

(A) Second generation CAR containing binding-, spacer and two activation domains. **(B)** Multi CAR-T cell expressing two CARs targeting different antigens. **(C)** Tandem or bi-specific CAR contains two binding domains which both require antigen binding to activate the T cell. **(D)** In conditional CAR-Ts, activation and co-activation domains are separated and conjugated to different extracellular binding domains. Only binding of both target antigens will lead to T cell activation. **(E)** Multi CAR-T technology containing one stimulatory and one inhibitory CAR. Binding of the target antigen by the inhibitory CAR inhibits the signal of the activated CAR. **(F)** Extracellular switch technology uses a small molecule required to bridge binding of the CAR domain and the target antigen. **(G)** Intracellular switch molecules need to be applied to transmit the co-stimulatory signal from the co-stimulatory to the activation domain. **(H)** CAR-T cells secreting cytokines or small molecules to overcome T cell inhibitory signals.

Another crucial point for T cell therapy against HIV examines the potential infection of the therapeutic T cells by the virus. Several approaches have been undertaken to prevent anti-HIV CAR-T cells from HIV infection. One of the most promising technologies is the inhibition of HIV co-receptor CCR5 zinc finger nucleases (Tebas *et al.* 2014). Infusion of autologous CD4 T cells in which the CCR5 gene was rendered permanently dysfunctional by a zinc-finger nuclease (ZFN) was shown to be safe and efficacious. However, disabling of CCR5 fusion is efficacious only against HIV strains with CCR5 tropism, whereas CXCR4 remains a potential co-receptor for viral cell entry. Handling, activation and expansion of potentially infected T cells *ex vivo*, before reinfusion into the patient, bears the risk of viral spread within the therapeutic product. Manufacturing of CAR-T cells has improved significantly, enabling provision of safe and potent cell products (Wang and Riviere 2016). Safe and efficacious manufacturing of autologous T cell therapy products for HIV-positive patients has also been established. T cells showed good transduction efficiency and expansion *ex vivo* while viral replication is suppressed by ART (Sung *et al.* 2018). However, one very promising approach is the targeted transduction of T cells with lenti- or retroviral vectors *in vivo* (Pfeiffer *et al.* 2018). Hereby, the CAR transgene could be delivered to a specific T cells population to express CARs without the need of extensive handling an activation *ex vivo*.

Targeting the viral reservoir of HIV with anti-CD4-DARPin CAR-T cells is undoubtedly a very radical approach. Potential risks of toxicity towards neuronal tissues and insufficient recovery of $CD4^+$ T cells require extensive and careful investigation. However, the prospect of successful depletion of the latent viral reservoir, rendering further therapy unnecessary, is the aim strived for since discovery of HIV. Moreover, the hidden reservoirs have shown to be unassailable for any type of therapeutic agent. The Results of this thesis demonstrate the potential to deplete the latent HIV reservoir in a specific and efficient way. Further investigation and modification will be necessary to improve the concept of targeting CD4 with CAR-T cells. Efficacy, safety and efficiency of the approach are the main aspects to proof viability of this approach. New technologies are currently developed worldwide to expand capabilities of CAR-T cells, overcoming hurdles and caveats of these therapies. Enhancement of specificity, efficacy and persistence of CAR-T cells provide very promising prospects of achieving a sterilizing cure for HIV patients.

5 Summary

5.1 Summary figure

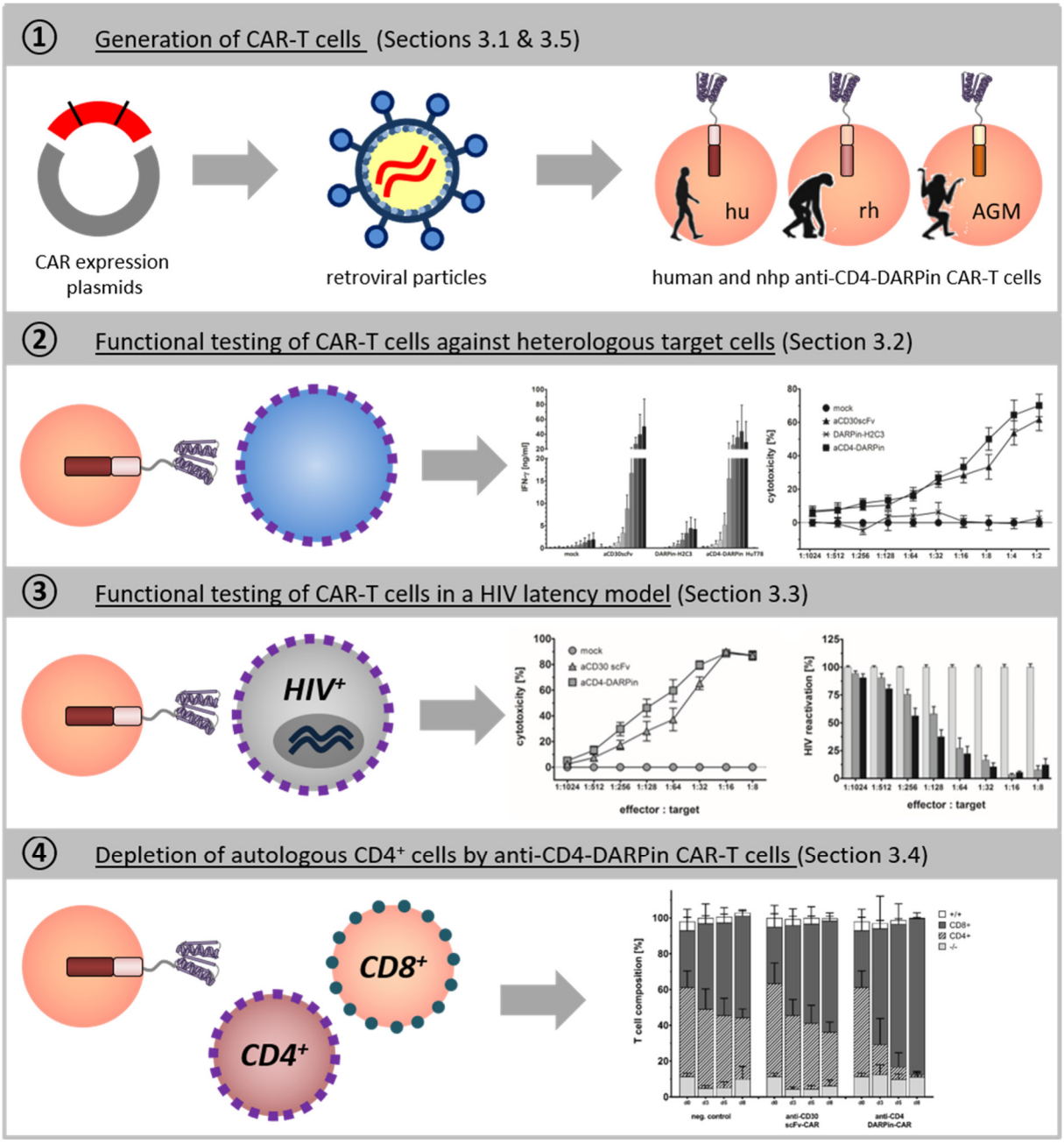

Figure 38: Overview of assays and results.

① Generation of human and non-human primate anti-CD4-DARPin CAR-T cells by transduction with gamma-retroviral particles. ② Functional testing of specific activation and cytotoxicity by co-culture with $CD4^+$ target cells and $CD4^-$ non-target cells. ③ Functional testing of CAR-T cells in an HIV-latency model, pre- and post-activation with LRAs (latency reversing agents). ④ Cytotoxicity of human and non-human primate CAR-T cells against autologous primary $CD4^+$ T cells.

5.2 Summary (English)

Specific targeting of HIV infected cells has been a challenge since the discovery of the virus. To date, antiretroviral therapy is capable of inhibition of viral spread and controlling the viral load at levels below detection limit. However, the latent viral reservoir persists even during intensive treatment, and produces new infectious viral particles as soon as medication is discontinued. But antiretroviral therapy is cost-intensive, causes major side effects and involves close monitoring of the patient. Relapse due to viral mutation may also require multiple changes in the medication strategy. It is therefore essential for a sustainable cure, to also fully eliminate the latent viral reservoir. Chimeric Antigen Receptor (CAR)-T cell therapy has been very successful in the treatment of hematologic malignancies. Autologous T cells of patients are genetically modified to express a chimeric receptor, which enables them to specifically bind their target antigen and deplete the target cell without the need of MHC-presentation. Transferring this CAR-T cell technology from targeting of malignant B cells to HIV-infected T cells could potentially be the way to a sterilising cure. However, the development of anti-HIV CAR-T cells has been dormant in the past years due to lack of efficiency, and because targeting of viral proteins on the surface of infected cells did not deplete the latent viral reservoir, hence did not show any improvement compared to conventional antiretroviral therapy. Fast progress in recent development of next generation CARs led to significant enhancement of efficacy and specificity. In addition to that, targeting of cellular rather than viral proteins could potentially overcome persistence of the latent reservoir. Therefore, CD4 as the exclusive HIV entry receptor into the host cell could be a suitable cellular target for anti-HIV CAR-T cells, because all infected cells express CD4, including the latent reservoir.

One aim of this thesis was to investigate whether CAR-T cells can specifically and efficiently deplete their CD4-positive target cell population. Therefore, a second-generation CAR was utilised, bearing CD3zeta and CD28 co-stimulatory domains, as successfully used in clinical trials. A CD4-specific Designed Ankyrin Repeat Protein (DARPin) was used as a binding domain, since this molecule was shown to be small, well expressed on the cell surface, specific and not immunogenic. Generation of an expression plasmid, encoding all CAR domains in a cassette system, provided a platform for flexible and individual CAR design. Transduction of primary human T cells with gamma-retroviral particles led to CAR expression on the cell surface. Co-culture resulted in dose-dependent CAR-mediated T cell activation and cytotoxicity towards target but not towards non-target cells, verifying specificity and potency of anti-CD4-DARPin CAR-T cells. High efficiency was shown during co-culture with non-target cells including decreasing amounts of target cells. CD4 specific cytotoxicity was also observed against very low target cell levels. Human CD4 was selected as a target because it is the entry receptor for HIV into the host cell, and therefore expressed on all HIV-positive cells, irrespectively of

their activation state. Another question of this thesis was therefore, if CD4-specific CAR-T cells have the potential to deplete the CD4-positive T cell compartment and thereby also the latent HIV reservoir. Anti-CD4-DARPin CAR-T cells showed efficient and specific cytotoxicity against an HIV-latency model - towards activated, as well as latently infected cells with low levels of target expression. Furthermore, CD4 T cells were efficiently depleted in an autologous setup, without major impact on the CD4-negative T cell population. Taken together, anti-CD4-DARPin CAR-T cells have shown specificity, efficiency and potency, the main properties, required for successful CAR-T cell therapy. With regard to further investigation of this approach in an immunocompetent animal model, anti-CD4-DARPin CARs were not only expressed on human, but also non-human primate T cells. Moderate depletion of autologous CD4 target cells was observed using T cells of rhesus macaques, providing the groundwork to test this approach in a well-established immunocompetent animal model.

In summary, the results of this work provide evidence, that anti-CD4-DARPin CAR-T cells are a promising approach to specifically deplete potential HIV host cells, along with elimination of latently infected cells. This was achieved by combination of a potent 2nd generation CAR, driving efficient T cell activation with a specific and well expressed DARPin binding domain, targeting a cellular protein to capture all potentially HIV-positive cells, including those in a latent stage. These data provide a basis for further investigation of anti-CD4-DARPin CAR-T cells for HIV therapy.

5.3 Zusammenfassung (deutsch)

Seit der Entdeckung des HI-Virus stellt das spezifische Targeting infizierter Zellen eine der größten Herausforderungen dar. Antiretrovirale Therapie ist mittlerweile in der Lage, die Verbreitung des Virus im Patienten zu inhibieren und die Viruslast unter dem Detektionslimit zu halten. Allerdings bleibt das latente Virusreservoir trotz intensiver Therapie bestehen und beginnt mit der Produktion neuer infektiöser Partikel, sobald die Medikation eingestellt wird. Dauerhafte antiretrovirale Therapie ist jedoch kostenintensiv, verursacht häufig starke Nebenwirkungen und bedarf einer engmaschigen Überwachung der Patienten. Rückfälle aufgrund von Virusmutationen können auch mehrfach Änderungen der Medikationsstrategie erforderlich machen. Um eine nachhaltige Heilung zu erreichen ist es daher essentiell, auch das latente virale Reservoir vollständig zu auszulöschen. Die Therapie mit Chimeric Antigen Receptor (CAR)-T Zellen ist derzeit äußerst erfolgreich in der Behandlung von Blutkrebs. Autologe T-Zellen von Patienten werden hierfür genetisch modifiziert um einen chimären Rezeptor zu exprimieren, der diese dazu befähigt, spezifisch an ihr Zielantigen zu binden und die Zielzelle zu zerstören, ohne dass das Zielantigen dafür auf dem MHC-Komplex präsentiert werden muss. Die Übertragung dieser CAR-T-Zell-Technologie von der Behandlung maligner B-Zellen auf HIV-infizierte T-Zellen könnte potentiell der Weg zu einer vollständigen Heilung sein. Allerdings ist die Entwicklung von anti-HIV CAR-T-Zellen in den letzten Jahren kaum vorangeschritten. Gründe hierfür waren vor allem mangelnde Effizienz, sowie der Umstand, dass das Ansteuern viraler Proteine auf der Oberfläche infizierter Zellen das latente virale Reservoir nicht einschließt. Verglichen mit herkömmlicher antiretroviraler Therapie ergab sich dadurch kein nennenswerter Vorteil der CAR-T-Zellen. Mit der Entwicklung von CARs fortgeschrittener Generationen, wurden Wirksamkeit und Spezifität enorm verbessert. Zusätzlich dazu, könnte die Wahl eines zellulären statt eines viralen Zielproteins möglicherweise auch das latente virale Reservoir eliminieren. Weil CD4 der exklusive Eintrittsrezeptor für HIV in die Wirtszelle ist und somit alle infizierten Zellen, inclusive des latenten Reservoirs, CD4 exprimieren, könnte dieser ein passendes Zielantigen für anti-HIV CAR-T-Zellen sein.

Ein Ziel dieser Dissertation war es, herauszufinden ob CAR-T-Zellen in der Lage sind, ihre CD4-positive Zielzellpopulation effizient zu depletieren. Hierfür wurde ein CAR der zweiten Generation mit CD3zeta- und CD28 Co-Aktivierungsdomäne genutzt, wie auch in verschiedensten erfolgreichen klinischen Studien. Ein CD4-spezifisches Designed Ankyrin Repeat Protein (DARPin) wurde als Bindedomäne eingesetzt. Dieses Molekül gilt als klein, gut auf Zelloberflächen zu exprimieren, spezifisch und nicht immunogen. Die Herstellung eines Expressionsplasmids, welches alle CAR-Domänen in einem Kassettensystem codiert, lieferte die Plattform für flexibles und individuelles Design verschiedener CARs. Transduktion primärer humaner T-Zellen mit Gamma-Retroviralen Transfervektorpartikeln führte schließlich zur CAR-

Expression auf deren Zelloberfläche. Co-Kultivierung führte zu dosisabhängiger, CAR-vermittelter T-Zell-Aktivierung und Zytotoxizität gegenüber antigen-positiven, nicht aber gegenüber antigen-negativen Zielzellen. Somit konnten Spezifität und Wirksamkeit von anti-CD4-DARPin CAR-T-Zellen verifiziert werden. Eine hohe Effizienz zeigte sich auch während der Kultivierung mit antigen-negativen Zellen, denen antigen-positive Zielzellen in abnehmender Konzentration beigefügt wurden. CD4-spezifische Zytotoxizität war auch dann noch gegeben, wenn die Zielzellen nur in sehr geringer Menge vorhanden waren.

Humanes CD4 wurde als Zielantigen ausgewählt, weil es als Eintrittsrezeptor in die Wirtszelle fungiert und somit auf allen HIV-positiven Zellen exprimiert wird, unabhängig von deren Aktivierungszustand. Ein weiteres Ziel dieser Arbeit war daher, festzustellen ob CD4-spezifische CAR-T-Zellen in der Lage sind, mit der CD4-positiven Zellpopulation auch das latente virale Reservoir zu depletieren. Anti-CD4-DARPin CAR-T-Zellen zeigten effiziente und wirksame Zytotoxizität gegenüber einem HIV-Latenzmodell – sowohl im aktivierten, als auch im latenten Zustand mit geringer Antigenexpression. Darüber hinaus wurden auch autologe CD4 T-Zellen effizient depletiert, ohne erkennbare Auswirkungen auf die CD4-negative T-Zellpopulation. Insgesamt zeigten anti-CD4-DARPin CAR-T-Zellen Spezifität, Effizienz und Wirksamkeit – die Haupteigenschaften für eine erfolgreiche CAR-T-Zell-Therapie. Um diesen Ansatz in einem immunkompetenten Tiermodell weiter zu untersuchen, wurden anti-CD4-DARPin CARs nicht nur in humanen, sondern auch in T-Zellen von nicht-humanen Primaten exprimiert. Autologe CD4-Zielzellen von Rhesusaffen wurden im mittleren Maße depletiert und dienen als Grundlage, um diesen Ansatz in einem etablierten immunkompetenten Tiermodell zu testen.

Die Ergebnisse dieser Dissertation zeigen, dass anti-CD4-DARPin CAR-T-Zellen ein vielversprechender Ansatz sind, gezielt potenzielle HIV Wirtszellen zu depletieren und dabei auch latent infizierte Zellen zu eliminieren. Dies wurde erreicht durch die Kombination eines potenten CAR der zweiten Generation, welcher T-Zellen effizient mittels einer gut exprimierten DARPin-Bindedomäne aktiviert. Gleichzeitig werden durch das Ansteuern eines zellulären Zielproteins alle potenziell HIV-positiven Zellen erfasst, inklusive derer, die sich im Stadium der Latenz befinden. Diese Daten bieten eine Grundlage zur weiteren Erforschung von anti-CD4-DARPin CAR-T Zellen zur Behandlung von HIV.

6 Literature

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7 Abbreviations

°C	*degree Celsius*
A	*ampere*
AFP	*Alpha-Fetoprotein*
AGM	*African Green Monkey*
AIDS	*acquired immune deficiency syndrome*
ALL	*Acute lymphoblastic leukaemia*
Amp	*ampicillin*
AP-1	*activator protein 1*
APC	*allophycocyanin*
APC	*antigen-presenting cell*
APOBEC	*apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like*
AR	*antigen receptor*
ATCC	*American Type Culture Collection*
BCMA	*B-cell maturation antigen*
BiTE	*Bispecific T cell Engager*
bNAbs	*Broadly Neutralizing HIV-1 Antibodies*
bp	*base pairs*
BSA	*bovine serum albumin*
ca.	*circa*
CAIX	*Carbonic Anhydrase IX*
cap	*capsid protein*
CAR	*chimeric antigen receptor*
cART	*combination antiretroviral therapy*
Cas	*CRISPR associated protein*
CCR5	*C-C chemokine receptor type 5*
CD	*cluster of differentiation*
CDC	*centers of disease conrol*
CDK9	*cyclin-dependent kinase 9*
cDNA	*complementary DNA*
CEA	*carcinoembryonic antigen*
CFSE	*carboxyfluorescein succinimidyl ester*
CLL	*chronic lymphocytic leukaemia*
cMET	*c-terminal MET*
CMV	*cytomegalovirus*
CNS	*central nervous system*
CRD	*carbohydrate recognition domain*
CRISPR	*clustered regularly interspaced short palindromic repeats*
CRS	*cytokine release syndrome*
CTL	*cytotoxic T lymphocyte*
ctrl	*control*
CxCR4	*C-X-C chemokine receptor type 4*
Da	*Dalton*
DARPin	*designed ankyrin repeat protein*
DART	*Dual-Affinity Re-Targeting protein*
dd	*double-distilled*
DMEM	*Dulbecco's modified eagle medium*

DMSO	*dimethyl sulfoxide*
DNA	*deoxyribonucleic acid*
dNTP	*deoxynucleoside triphosphate*
DPBS	*Dulbecco's phosphate buffered saline*
DRB	*5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole*
DSIF	*DRB sensitivity inducing factor*
DTT	*dithiothreitol*
E. coli	*Escherichia coli*
e.g.	*exempli gratia, for example*
E:T	*effector:target*
EDTA	*ethylenediaminetetraacetic acid*
eGFP	*enhanced GFP*
EGFR	*Epidermal Growth Factor Receptor*
ELISA	*enzyme-linked immunosorbent assay*
EMA	*European medicines association*
Env	*envelope protein*
EpCAM	*epithelial cell adhesion molecule*
ER	*endoplasmic reticulum*
ErbB2	*Receptor tyrosine-protein kinase ErbB2*
et al.	*and others*
Fab	*fragment antigen binding*
FACS	*fluorescence-activated cell sorting*
FBS	*fetal bovine serum*
Fc	*fragment crystallizable*
FCS	*fetal calf serum*
FDA	*US Food and Drug Administration*
fDC	*follicular dendritic cells*
FITC	*fluorescein isothiocyanate*
FL	*fluorescent light*
for	*forward*
FSC	*forward scatter*
g	*gram*
gag	*group-specific antigen, gene in retroviruses*
GaLV	*Gibbon ape leukaemia virus*
GD2	*ganglioside 2D*
GFP	*green fluorescent protein*
Gp	*glycoprotein*
GPC	*oncofetal antigen glypican*
h	*hour*
HCl	*hydrochloride*
HEK	*human embryonic kidney*
Her2	*human epidermal growth factor receptor 2*
His	*hexa-histidine tag*
HIV	*human immunodeficiency virus*
HLA	*human leukocyte antigen*
HRP	*horseradish peroxidase*
HSC	*hematopoietic stem cell*
hu	*human*

IF	*immunofluorescence*
IFN	*interferon*
Ig	*immunoglobulin*
IgG	*immunoglobulin G*
IL	*interleukin*
IN	*integrase*
INSTI	*integrase strand transfer inhibitor*
IRES	*internal ribosome entry site*
ITAM	*immunoreceptor tyrosine-based activation motif*
ITR	*inverted terminal repeat*
IU	*international unit*
K	*potassium*
k	*kilo or x1000*
Kana	*Kanamycin*
L	*liter*
LB	*Luria-Bertani*
LTR	*long terminal repeat*
LV	*lentiviral vector*
M	*mol(ar)*
M	*meter*
m	*milli-*
mAb	*monoclonal antibody*
mDC	*myeloid dendritic cell*
MFI	*Mean fluorescence intensity*
MHC	*major histocompatibility complex*
min	*minute*
MIP	*macrophage Inflammatory Proteins*
miRNA	*microRNA*
MLV	*murine leukaemia viruses*
MLV	*murine leukaemia virus*
MOI	*multiplicity of infection*
MOPS	*3-(N-morpholino)propanesulfonic acid*
mRNA	*messenger RNA*
MUC1	*mucine1*
MV	*measles virus*
n	*Nano-*
nef	*negative regulatory factor*
NELF	*negative elongation factor*
NES	*nuclear export signal*
NF-AT	*nuclear factor of activated T-cells*
NFκB	*nuclear factor kappa-light-chain-enhancer of activated B cells*
nhp	*non-human primate*
NK	*natural killer cell*
NKG2D	*natural killer group 2D*
NNRTI	*non-nucleoside reverse transcriptase inhibitor*
NRTI	*nucleoside reverse transcriptase inhibitor*
PBMC	*peripheral blood mononuclear cell*
PBS	*phosphate buffered saline*

PCR	*polymerase chain reaction*
PD-1	*programmed cell death 1 protein*
pDC	*plasmacytoid dendritic cell*
PE	*phycoerythrin*
PEI	*Polyethylenimine or Paul-Ehrlich-Institut*
PEP	*post-exposure prophylaxis*
PES	*polyethersulfone*
PFA	*paraformaldehyde*
PLL	*Poly-L-Lysine*
PML	*promyelocytic leukaemia*
pol	*DNA-polymerase, gene in retroviruses*
Pol II	*RNA polymerase II*
PrEP	*pre-exposure prophylaxis*
PSMA	*prostate-specific membrane antigen*
pTEF	*positive elongation factor*
PVDF	*polyvinylidene difluoride*
qPCR	*quantitative PCR*
rep	*viral regulatory proteins*
rev	*regulator of expression of virion proteins*
rev	*reverse*
Rh	*rhesus*
RNA	*ribonucleic acid*
rpm	*rounds per minute*
RPMI	*Roswell Park Memorial Institute*
RRE	*reverse response element*
RT	*reverse transcription*
RT-PCR	*reverse transcription PCR*
s	*second*
S.O.C.	*super optimal broth with catabolite repression*
SAHA	*suberoylanilide hydroxamic acid*
SB	*sleeping beauty*
scFv	*single-chain Fragment variable*
SCID	*severe combined immunodeficiency*
SD	*standard deviation*
SDS	*sodium dodecyl sulfate*
SDS-PAGE	*SDS polyacrylamide gel electrophoresis*
sec	*second*
SEM	*standard error of the mean*
siRNA	*small interfering RNA*
SIV	*simian immunodeficiency virus*
SLP-76	*SH2 domain containing leukocyte protein of 76kDa*
SOB	*super optimal broth*
SSC	*side scatter*
t.u.	*transducing units*
TAR	*trans-activation region*
tat	*HIV trans-activator*
TBS-T	*tris-buffered saline Tween*
TCR	*T cell receptor*

TD	*targeting domain*
TEMED	*tetramethylethylendiamine*
T_H	*helper T cell*
TL	*transmitted light*
Tm/T_M, T_{CM}	*memory T cell, central memory T cell*
TMB	*3,3',5,5'-Tetramethylbenzidine*
TMD	*transmembrane domain*
TNF	*tumour necrosis factor*
TNFRSF	*tumour necrosis factor receptor superfamily*
Treg	*regulatory T cell*
TRIM5α	*Tripartite motif-containing protein 5, isoform α*
TRIS	*tris(hydroxymethyl)aminomethane*
T_{TM}	*translational memory T cell*
U	*unit*
USA	*United States of America*
UV	*ultraviolet*
V	*volt*
VEGFR	*Vascular Endothelial Growth Factor Receptor*
V_H	*variable region of the antibody heavy chain*
vif	*viral infectivity factor*
V_L	*variable region of the antibody light chain*
VP	*viral protein*
vpr	*viral protein R*
vpu	*viral protein U*
vpx	*viral protein X*
W	*watt*
w/o	*without*
WB	*Western Blot*
WHO	*World Health Organization*
WPRE	*Woodchuck Hepatitis virus posttranscriptional regulatory element*
wt	*wild type*
xg	*relative centrifugal force*
ZFNs	*zinc-finger nucleases*
α	*alpha*
a/A	*alpha or anti*
β	*beta*
B	*beta*
γ	*gamma*
Γ	*gamma*
δ	*delta*
Δ	*delta*
ε	*epsilon*
ζ	*zeta*
κ	*kappa*
λ	*lambda*
μ	*mu, micro-*
ψ	*psi*

7.1 Amino acids

A	*Ala*	*Alanine*	M	*Met*	*Methionine*
C	*Cys*	*Cysteine*	N	*Asn*	*Asparagine*
D	*Asp*	*Aspartic acid*	P	*Pro*	*Proline*
E	*Glu*	*Glutamic acid*	Q	*Gln*	*Glutamine*
F	*Phe*	*Phenylalanine*	R	*Arg*	*Arginine*
G	*Gly*	*Glycine*	S	*Ser*	*Serine*
H	*His*	*Histidine*	T	*Thr*	*Threonine*
I	*Ile*	*Isoleucine*	V	*Val*	*Valine*
K	*Lys*	*Lysine*	W	*Trp*	*Tryptophan*
L	*Leu*	*Leucine*	Y	*Tyr*	*Tyrosine*

7.2 Nucleobases

A	*Adenine*
C	*Guanine*
G	*Cytosine*
T	*Thymine*
U	*Uracil*

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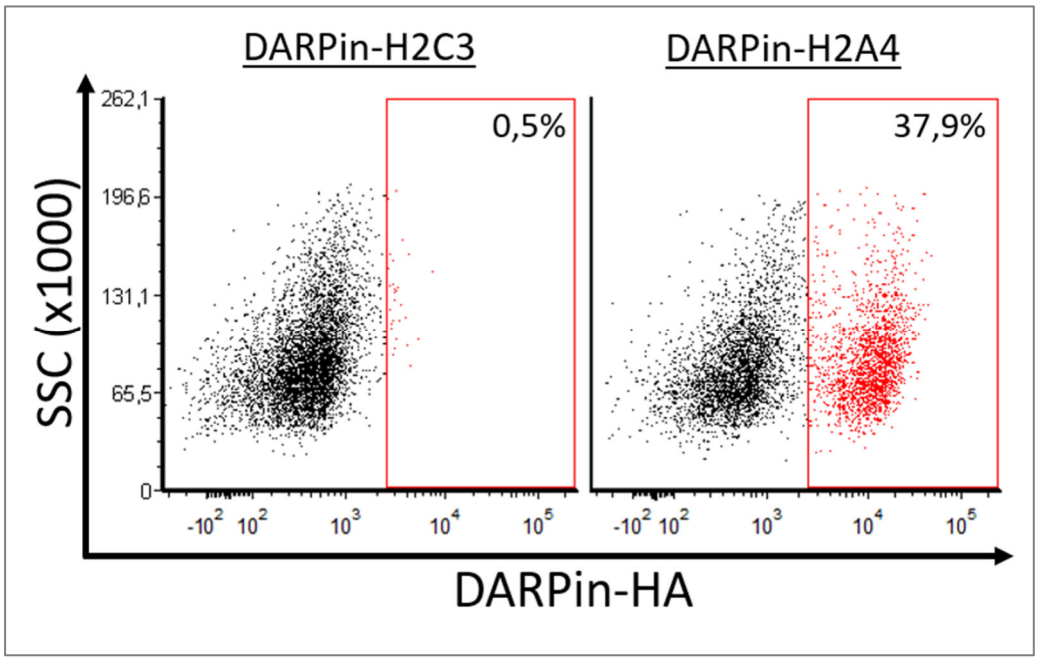
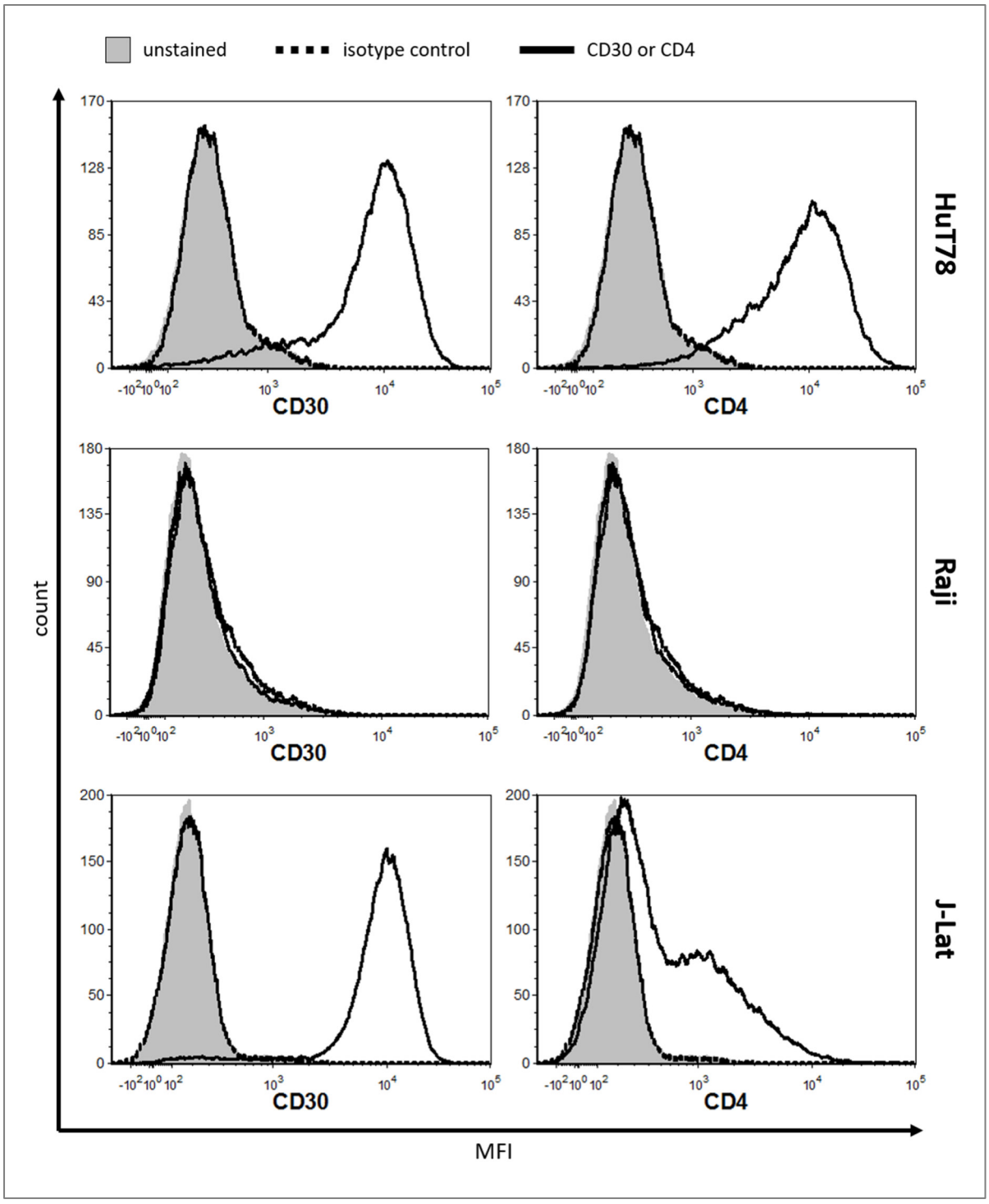
9 Appendix

9.1 Validation of unspecific control DARPin domain and target cell lines



Supplementary Figure 1: Binding of DARPin to human PBMCs.

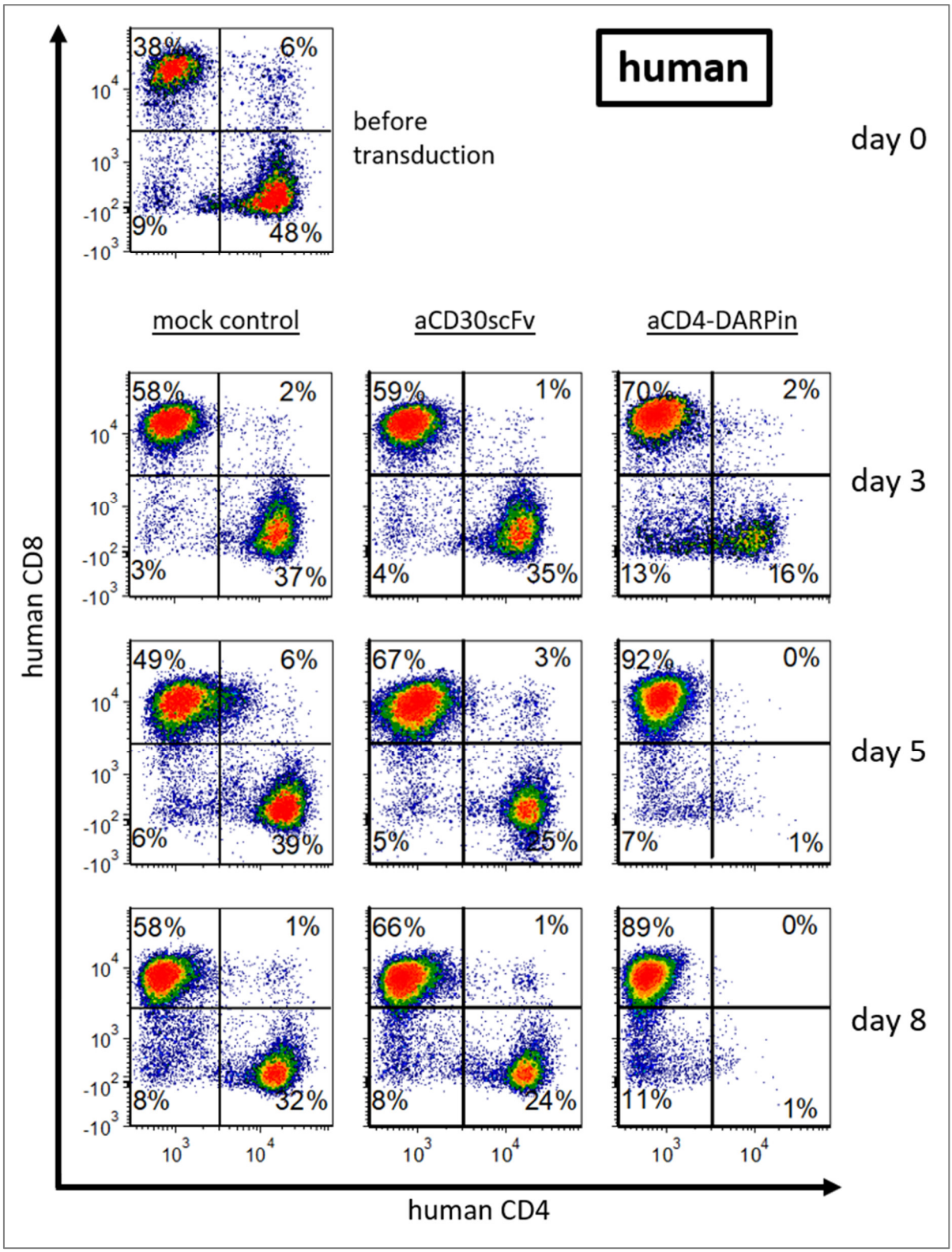
After incubation with human PBMCs, DARPin-associated HA-tag could be detected for positive con-trol DARPin_H2A4 but not for DARPin_H2C3. Raw data were kindly provided by Jessica Hartmann (depart-ment Pr1/PEI) who also performed this DARPin binding assay.



Supplementary Figure 2: Surface expression of target molecules on HuT78, Raji and J-Lat cells.

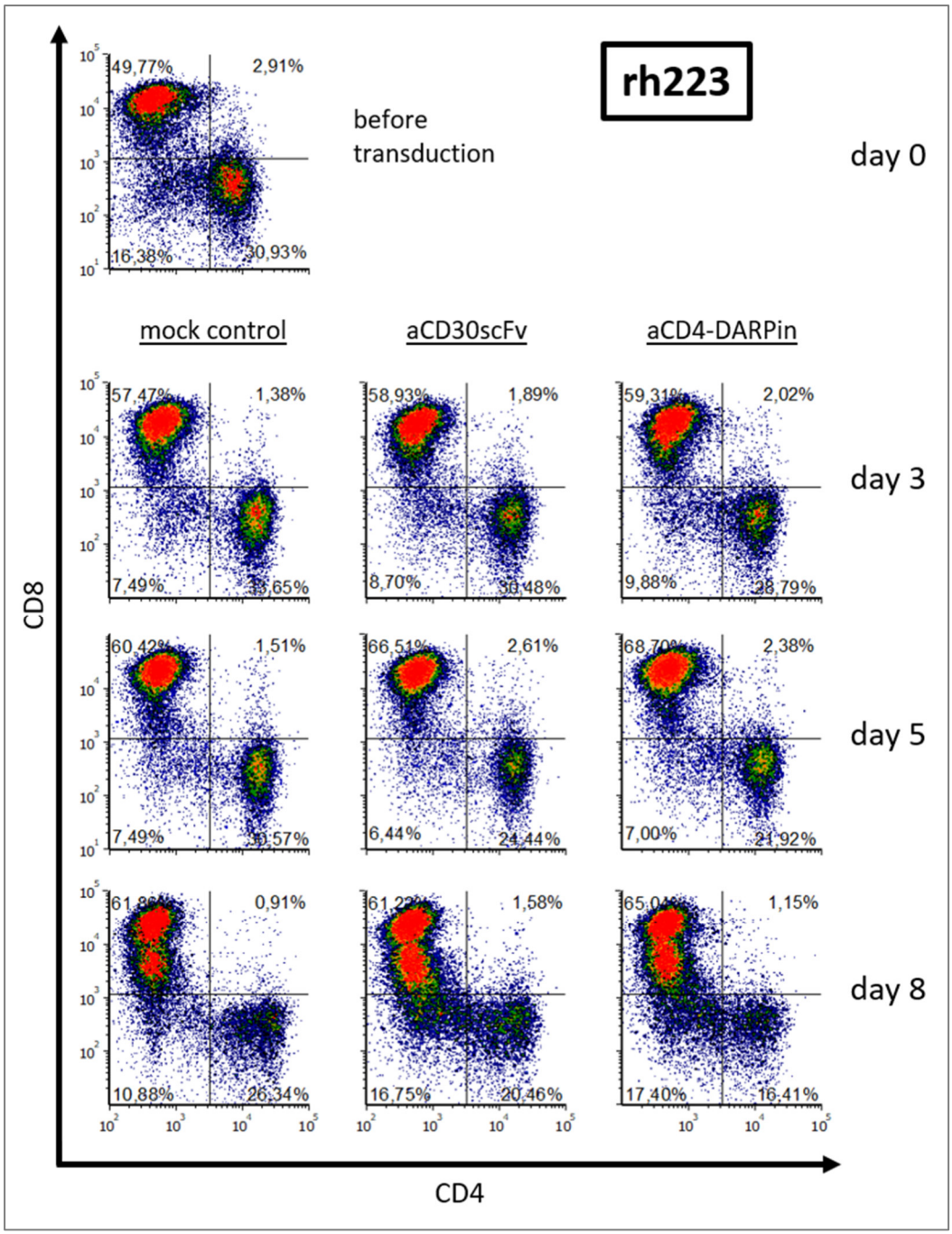
Expression of CAR target receptors human CD30 and human CD4 on the surface of target cell lines HuT78, Raji and J-Lat, assessed by flow cytometry. Unstained samples are shown in solid grey, isotype controls in dotted lines and antibody stains in black solid lines. Displayed is cell count against MFI (Mean Fluorescence Intensity) of PE antibody signals.

9.2 Depletion of autologous CD4⁺ T cells – detailed results



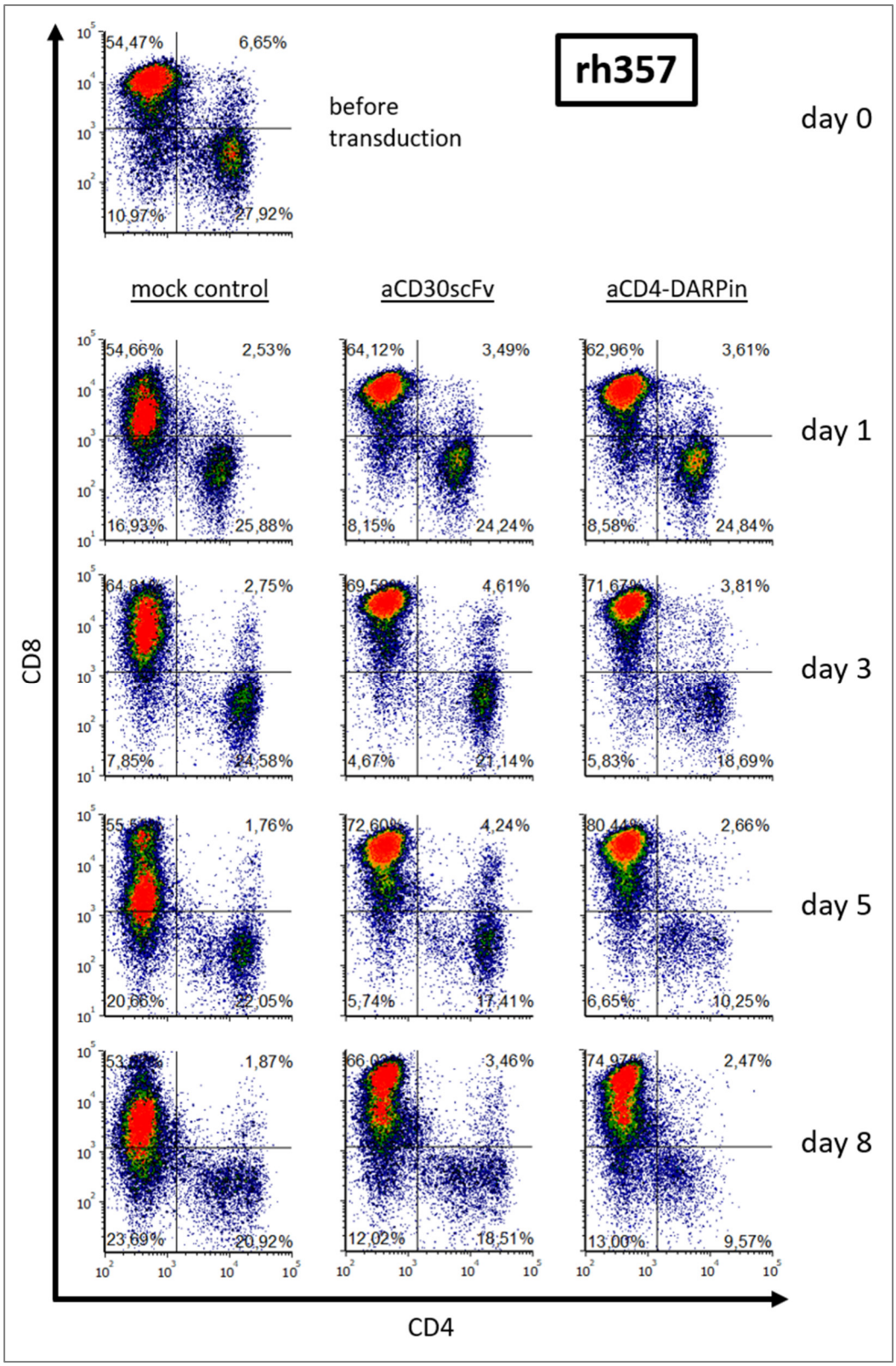
Supplementary Figure 3: Detailed presentation of depletion of autologous human CD4⁺ T cells.

T cells were stained for T cell markers CD3, CD4 and CD8 before transduction with retroviral vectors, as well as on days 3, 5 and 8 post transduction. Within the CD3⁺ T cell population, it was distinguished between CD4 and CD8 expressing cells. Shown here is one representative donor of six.



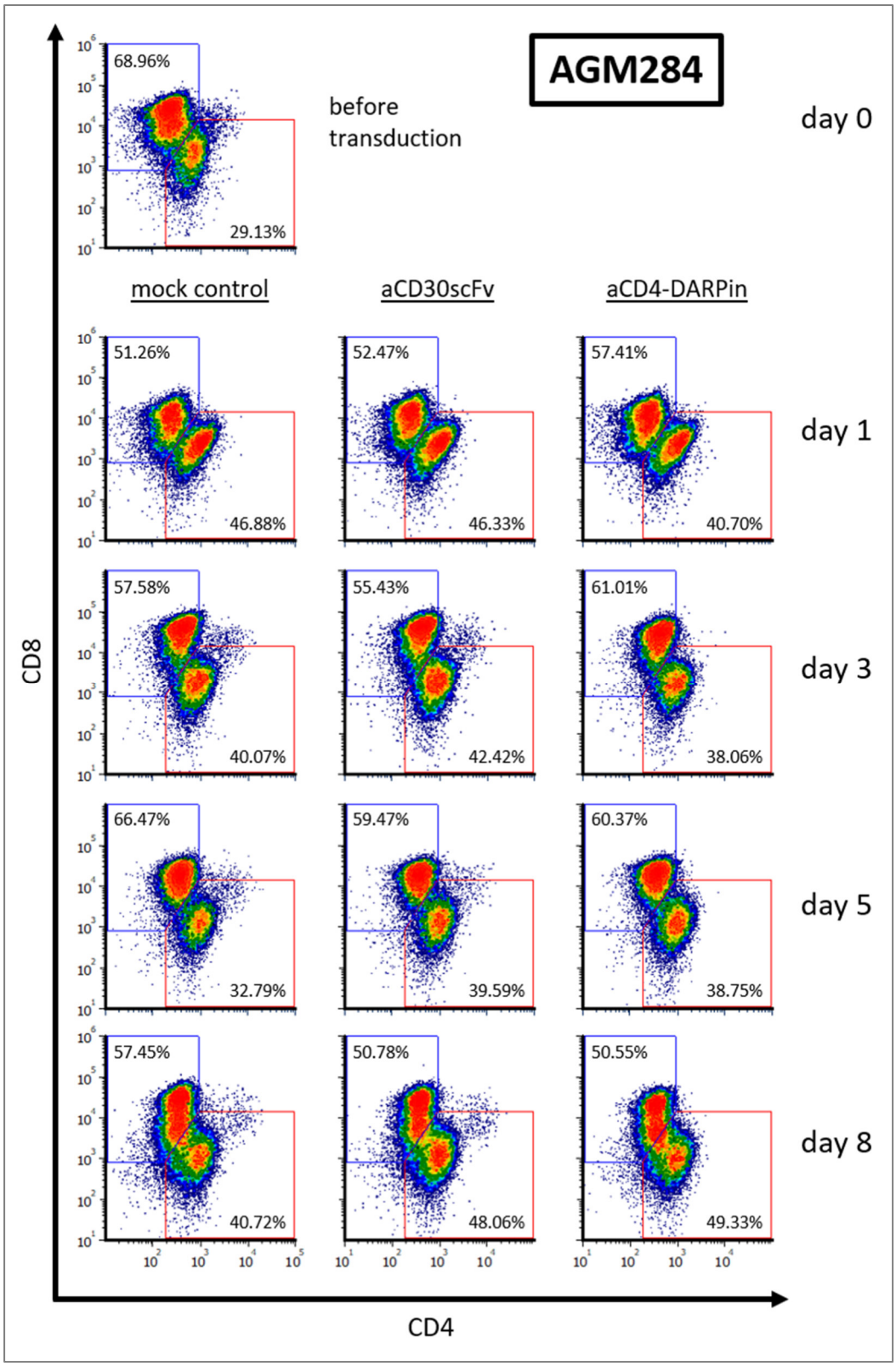
Supplementary Figure 4: Detailed presentation of depletion of autologous CD4⁺ T cells of rhesus223.

T cells were stained for T cell markers CD3, CD4 and CD8 before transduction with retroviral vectors, as well as on days 1, 3, 5 and 8 post transduction. Staining with rhesus cross-reactive and fluorescently labelled antibodies was analysed in flow cytometry. Within the T cell population, it was distinguished between CD4 and CD8 expressing cells.



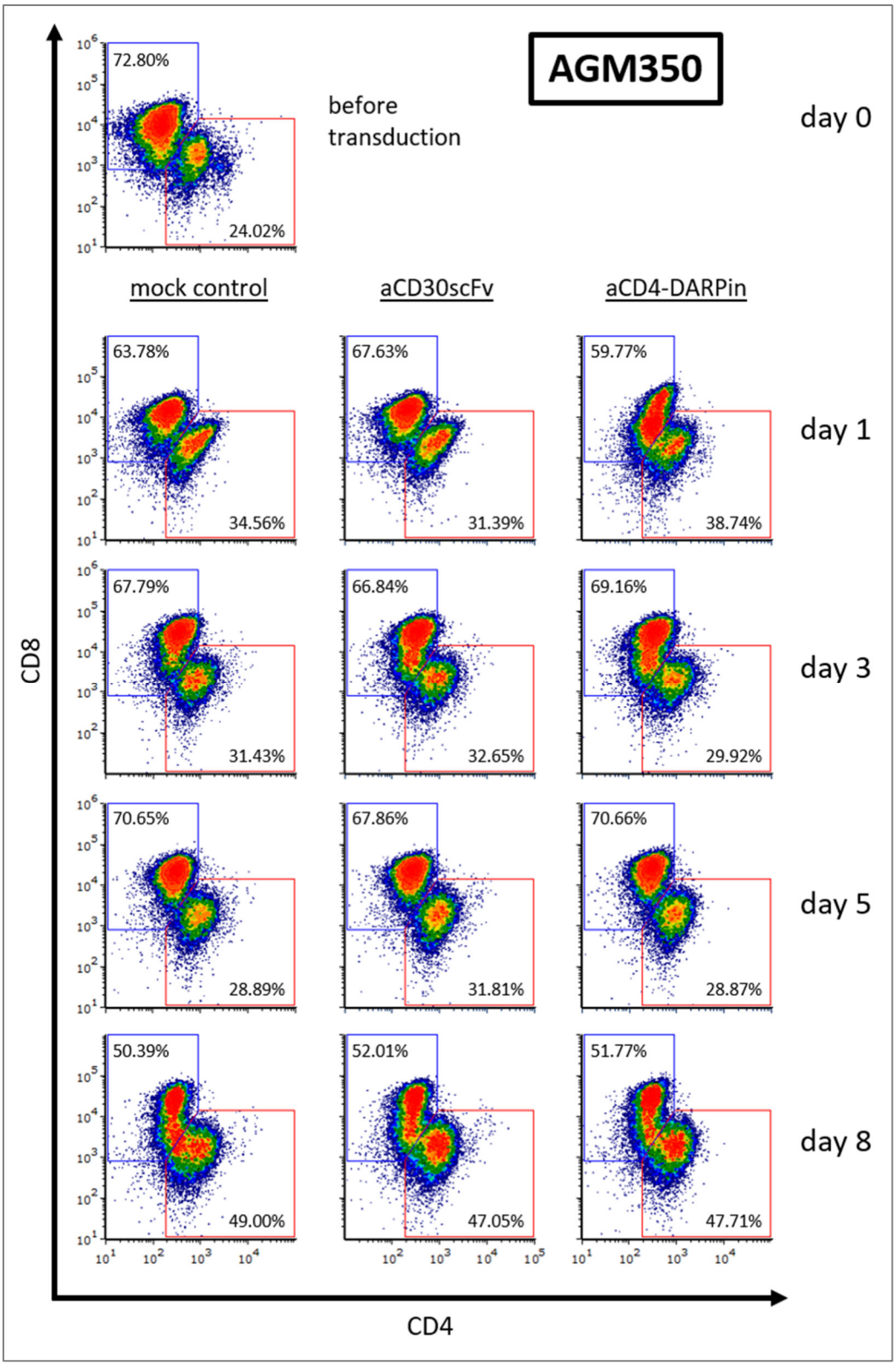
Supplementary Figure 5: Detailed presentation of depletion of autologous CD4$^+$ T cells of rhesus357.

T cells were stained for T cell markers CD3, CD4 and CD8 before transduction with retroviral vectors, as well as on days 1, 3, 5 and 8 post transduction. Staining with rhesus cross-reactive and fluorescently labelled antibodies was analysed in flow cytometry. Within the T cell population, it was distinguished between CD4 and CD8 expressing cells.



Supplementary Figure 6: Detailed presentation of depletion of autologous CD4$^+$ T cells of AGM284.

T cells were stained for T cell markers CD3, CD4 and CD8 before transduction with retroviral vectors, as well as on days 1, 3, 5 and 8 post transduction. Staining with rhesus cross-reactive and fluorescently labelled antibodies was analysed in flow cytometry. Within the T cell population, it was distinguished between CD4 and CD8 expressing cells.



Supplementary Figure 7: Detailed presentation of depletion of autologous CD4$^+$ T cells of AGM350.

T cells were stained for T cell markers CD3, CD4 and CD8 before transduction with retroviral vectors, as well as on days 1, 3, 5 and 8 post transduction. Staining with rhesus cross-reactive and fluorescently labelled antibodies was analysed in flow cytometry. Within the T cell population, it was distinguished between CD4 and CD8 expressing cells.

10 Statements

Parts of this thesis are included in following manuscripts:

Patasic *et al*; *"Designed Ankyrin Repeat Protein (DARPin) targets Chimeric Antigen Receptor (CAR) redirected T cells towards autologous CD4+ T cells."* (manuscript in preparation)

update to above: Patasic *et al*; *"Designed Ankyrin Repeat Protein (DARPin) to target chimeric antigen receptor (CAR) redirected T cells towards CD4$^+$ T cells to reduce the latent HIV$^+$ cell reservoir."* (manuscript accepted 16.07.2020 by Medical Microbiology and Immunology)

11 Presentations

11.1 Oral presentations

2016: *"DARPins as CAR binding domains"* Paul-Ehrlich-Institut, Annual Research Retreat, Ronneburg (Germany)

11.2 Poster presentations

2016: *"DARPins as novel CAR binding domains"* CAR-TCR Summit, Boston (USA)

2016: *"DARPins as novel CAR binding domains"* 26th Annual Meeting of the Society for Virology, Münster (Germany)

2015 *"Establishment of a non-human primate CAR-T cell model"* 25th Annual Meeting of the Society for Virology, Bochum (Germany)

2015 *"Establishment of a non-human primate CAR-T cell model"* 13th Annual Meeting Association for Cancer Immunotherapy (CIMT), Mainz (Germany)

2015 *"Establishment of a non-human primate CAR-T cell model"* Paul-Ehrlich-Institut, Annual Research Retreat, Heidelberg (Germany)

2014: *"Establishment of a non-human primate CAR-T cell model"* Paul-Ehrlich-Institut, Annual Research Retreat, Heidelberg (Germany)

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13 Curriculum vitae

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May 2019:	Submission of PhD Thesis, Department of Biology, TU Darmstadt Title: "*DARPin-targeted Chimeric Antigen Receptor T cells: CD4 as a cellular target shows potential to evade HIV latency reservoir.*"
Since February 2017:	Senior Scientist/Investigator at Oncology Cell Therapy Research Unit, GlaxoSmithKline, Stevenage, United Kingdom
June 2013 – October 2016:	PhD student at Paul-Ehrlich-Institut Langen, Junior Research Group 3 "Host-Pathogen Interactions"
June 2012 – May 2013:	Diploma Thesis at Paul-Ehrlich-Institut Langen, President's Research Group 2
October 2004 – June 2013:	Biology student at Johannes Gutenberg Universität, Mainz; Degree: Diplom Biologin
June 2004:	Allgemeine Hochschulreife at Gymnasium Gernsheim

14 Ehrenwörtliche Erklärung

Ich erkläre hiermit ehrenwörtlich, dass ich die vorliegende Arbeit entsprechend den Regeln guter wissenschaftlicher Praxis selbstständig und ohne unzulässige Hilfe Dritter angefertigt habe.
Sämtliche aus fremden Quellen direkt oder indirekt übernommenen Gedanken sowie sämtliche von Anderen direkt oder indirekt übernommenen Daten, Techniken und Materialien sind als solche kenntlich gemacht. Die Arbeit wurde bisher bei keiner anderen Hochschule zu Prüfungszwecken eingereicht.

Hiermit bestätige ich, dass diese elektronische Datei mit der schriftlichen Version der Dissertation, datiert auf den 10. Juni 2019, welche bei der TU Darmstadt eingereicht wurde, identisch ist.

Darmstadt, den 10. Juni 2019

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Lea Patasic